THE USE OF METABOLOMIC PROFILING TO ELUCIDATE MECHANISMS UNDERLYING ARSENIC-ASSOCIATED DIABETES

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ABSTRACT

Elizabeth Marie Martin: The use of metabolomic profiling to elucidate mechanisms underlying arsenic-associated diabetes
(Under the direction of Rebecca C. Fry)

Arsenic is a naturally occurring metalloid that is associated with numerous health effects. More than 100 million individuals are exposed globally to high levels of arsenic chronically through contaminated drinking water sources. Studies have shown these individuals are more likely to develop Diabetes Mellitus (DM). At present, the National Toxicology Program has classified chronic exposure to arsenic above 150 µg/L as a probable diabtogen. Furthermore, more recent epidemiological studies have highlighted that chronic arsenic exposure above 150 µg/L maybe associated with DM. While *in vitro* and *in vivo* studies have elucidated possible mechanisms by which arsenic induces diabetes, it is still unclear which of these mechanisms is fully relevant to humans. Metabolomic profiling could help provide key insights into metabolic alterations that occur in individuals exposed to arsenic that lead to diabetes. As metabolomics is an emerging field profiling both diabetic and non-diabetic individuals chronically exposed to arsenic could provide insight into metabolic alterations associated with chronic arsenic exposure, as well as insight into which of these alterations are associated with the development of diabetes.

The goal of this research was to identify metabolites associated with alterations in associated with arsenic exposure in diabetic and non-diabetic individuals in Chihuahua, Mexico. Using 492 unique metabolites in urine and plasma through an untargeted metabolomic screening, we assessed the relationship between these metabolites total urinary arsenic (U-tAs), a

commonly used measure of arsenic exposure, arsenic metabolism, as measured through speciated urinary arsenicals, and genotype of Arsenic +3 oxidation state methyltransferase (*AS3MT*), the main enzyme responsible for arsenic metabolism. We demonstrated that the identified metabolites are associated with alterations of key metabolic pathways including glucose metabolism, amino acid metabolism and vitamin B metabolism in association with arsenic exposure, arsenic metabolism and genotype of *AS3MT* in both diabetic and non-diabetic subjects. Many of the identified metabolites are associated with enzymes and metabolic pathways associated with the development of diabetes. Taken together, our research increase knowledge of mechanistic associations between arsenic-associated diabetes in humans.

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2HBG	2-Hour Blood Glucose after administration of a glucose tolerance test
AS3MT	Arsenic-3^+ oxidation state-methyltransferase (gene)
AS3MT	Arsenic-3^+ oxidation state-methyltransferase (protein)
BMI	Body Mass Index
DM	Diabetes Mellitus
(%)DMAs	(Percent) Dimethylated Arsenic in Urine
$DMAs^{3+}$	Trivalent Dimethylated Arsenic
$DMAs^{5+}$	Pentavalent Dimethylated Arsenic
DW-iAs	Drinking Water Inorganic Arsenic
iAs	Inorganic arsenic
(%)iAs	(Percent) Inorganic Arsenic in Urine
iAs^{3+}	Trivalent inorganic arsenic (Arsenite)
iAs^{5+}	Pentavalent inorganic arsenic (Arsenate)
$MMAs^{3+}$	Trivalent Monomethylated Arsenic
$MMAs^{5+}$	Pentavalent Monomethylated Arsenic
(%)MMAs	(Percent) Monomethylated Arsenic in Urine
ROS	Reactive Oxygen Species
SNP	Single Nucleotide Polymorphism
TCA	Tricarboxylic Acid (Krebs) Cycle
U-tAs	Urinary Total Arsenic
WHO	World Health Organization

INTRODUCTION

Type 2 Diabetes Mellitus (DM) is a metabolic disorder characterized by disruption of the insulin signaling pathway resulting in insulin resistance and pancreatic β-cell dysfunction and in limited uptake of glucose by cells. More than 641 million people are affected by type 2 diabetes globally, with between 17% and 40% unexplained by rises in obesity and other associated lifestyle factors [1]. Numerous studies have investigated the role of environmental contaminants in the development of diabetes [2, 3]. Of the chemicals investigated, the 2011 National Toxicology Program Workshop on diabetes and the Environment found sufficient evidence for the relationship between exposure to moderate and high ($\geq$ 150 µg iAs/L) levels of inorganic arsenic in drinking water and diabetes [4]. At the time, the Workshop deemed the evidence for association between moderate to low level (< 150 µg iAs/L) exposure to be insufficient [4].

Globally, more than 200 million people are exposed to inorganic arsenic at levels that exceed 10 µg As/L [5]. Of the numerous health effects associated with arsenic, including cancer of the skin and bladder, cardiovascular disease, stroke neurological effects and peripheral vascular disease [6], diabetes has been long studied, with several plausible mechanisms suggested by *in vivo* and *in vitro* laboratory studies [6, 7]. Such *in vitro* and *in vivo* studies have evaluated the impacts of arsenic on processes that govern glucose homeostasis, supporting that arsenic impairs insulin-dependent glucose uptake by inhibiting insulin-activated signal

transduction pathway, upregulates gluconeogenesis, and inhibits glucose-stimulated insulin secretion by pancreas [8].

The combined studies in this work investigate the mechanistic relationship between arsenic exposure and diabetes development through metabolomics profiling of humans, both diabetic and non-diabetic individuals, exposed to arsenic. These studies represent some of the first to assess alteration in the metabolome in relationship to arsenic exposure. The ultimate goal of these studies is to increase current knowledge surround mechanistic relationships by which arsenic may induce diabetes in humans, thus acting as a foundation for future research efforts in pinpointing the mechanism, development of therapeutic targets, and preventative strategies for arsenic-induced diabetes.

i. Sources of Arsenic Exposure

Arsenic is a naturally occurring inorganic metalloid that is ubiquitous across the globe. Humans are typically exposed through ingestion and inhalation. Dermal contact is a potential source of exposure as well however, very little is regarding how arsenic may enter the body through contact with the dermis, and health effects have only been reported in isolated occupational incidents. Inhalation exposure typically occurs in an occupational setting. However, it is also a component in tobacco smoke. More typically for the general population, ingestion is the primary route of exposure.

Arsenic is found in food stuffs and drinking water. Food stuffs commonly containing arsenic include seafood, and rice. Fish and shellfish are often the primary source of arsenic exposure in populations with low levels of arsenic in drinking water. However, arsenic found in seafood is predominately converted to arseno-betaine, a non-toxic arseno-sugar that is not linked

to detrimental health effects. In addition to seafood, arsenic has more recently been detected in other food stuffs. These include kale, wine, rice and chocolate. It is unclear what the health impacts of consumption of arsenic from these foods are as arsenic is present in metabolites different from those found in fish. Rice consumption is presently an area of active research as it is a staple crop for a large portion of the global population. Research efforts in this area have focused on breeding rice to take up less arsenic as well as irrigating crops with uncontaminated water. The health impacts of arsenic exposure through food is an active area of research.

Drinking water arsenic, primarily found in wells that are contaminated by natural sources like bedrock is the primary source of global arsenic toxicity. More than 100 million individuals are exposed above the WHO/USEPA limit of 10 µg/L globally. The most notable example of a large population exposed to high levels of arsenic from wells is East Bengal and Bangladesh, where approximately 20 million individuals are exposed above the Bangladesh MCL of 50 µg/L. Other countries, such as Mexico, Argentina, Twain and Thailand also have large populations experiencing high levels of arsenic due to contaminated drinking water. Furthermore, there are examples of exposure to arsenic in the United States for individuals who use private wells as drinking water sources. Given the range of exposure and large number of people exposure to arsenic, understanding arsenic toxicity and arsenic-induced disease is a global public health concern.

ii. Population-based studies of arsenic-associated diabetes mellitus

Population-based studies of arsenic-associated diabetes can typically be grouped into two study types: those examining the impacts of low exposure ($\leq$150 µg iAs/L), those examining the impacts of high exposure (>150 µg iAs/L) as defined by the NTP workshop [4]. To date, studies

at high exposure levels provide the most consistent results, typically finding a positive association between arsenic and DM. The studies of lower levels of exposure and occupational exposure report inconsistent results.

a. Studies of arsenic-associated diabetes at high levels of exposure (DW-iAs ≥ 150 µg/L)

A study of high levels of exposure in Taiwan was the first to identify the association between arsenic and diabetes [9]. Since then, there have been nine studies of exposures at high levels with three occurring in Bangladesh [10-12], five occurring in Taiwan [13-17], and one taking place in Cambodia [18] (Supplemental Table 1). Of all ten studies, seven have found associations between arsenic and diabetes in males and females [9, 11-16, 19], and one found associations between arsenic and diabetes in females [17]. Two studies found no association between arsenic and diabetes [10, 18] (Supplemental Table 1). These studies are difficult to compare directly as they did not use a common metric for diabetes diagnosis or arsenic exposure. Additionally, each study adjusted for different covariates. Arsenic exposure measures used were the cumulative exposure index (CEI) (n=5), direct drinking water measures (DW-iAs) (n=4), endemic vs. non-endemic regions (n=2), and the presence or absence of keratosis (skin lesions) (n=1) (Table 1). diabetes metrics used were self-reported (n=3), oral glucose tolerance testing (OGTT) (n=4), blood glucose levels, (n=4), glycosuria (n=2), HbA1c measurements (n=1), treatment history (n=1), death certificates (n=1) and insurance claims (n=1) (Supplemental Table 1). In terms of adjustment factors, two studies conducted were completely unadjusted [17, 18]. The remainder typically adjusted for age, sex, and/or BMI, with only two studies including additional factors of smoking status and education and physical activity (Supplemental Table 1). When considering the two studies that did not identify an association between arsenic and DM, Chen et al had a relatively small number of cases (n=241), despite the large size of the cohort

(n=11,319) [10], and the Huang et al study was a relatively small study (n=142 total) making it potentially underpowered [18]. While there has been some criticism of the eight studies that have detected an association [20, 21], they were all well powered, and include a prospective cohort study [14, 15]. Thus, overall, these studies suggest that arsenic and diabetes are linked

b. Studies of arsenic-associated diabetes at low levels of exposure (DW-iAs < 150 µg /L)

A total of 38 human population based studies have been conducted at lower levels of arsenic exposure (DW-iAs <150 µg/L). Of these studies 27 have found an association between arsenic levels and DM, and 11 have found no association between arsenic and diabetes (Supplemental Table 1). Unlike studies of high exposure that have taken place in a limited number of regions. Studies of lower exposures have occurred in Bangladesh [22, 23], Canada [24, 25], China [26-29], Cyprus [30], Denmark [31], Iran [32], Italy [33], Mexico [34-37], Serbia [38], South Korea [39-41], Spain [42], Taiwan [43], the United Kingdom [44] and the United States of America [45-58]. Similar to the studies of high exposures, different metrics of arsenic exposure, diabetes and adjustment factors were used. Arsenic exposure was determined by U-tAs for 15 studies, DW-iAs for eight studies, blood arsenic for three studies, CEI for three studies, geographic region based arsenic measures for three study, hair arsenic for two studies, time weighed average exposures for 2 studies and, exfoliated urothelial cells for one study. For determination of DM, the majority of studies (n=26) used either a biomarker measure (FBG $\geq$ 126, 2HBG $\geq$ 200, HbA1c $\geq$ 6.5, Oral Glucose Tolerance Test), whether a subjected used diabetes medication or physician diagnosis. The remaining studies used 12 studies used a variety of metrics, including death certificates and mortality registries (n=4), hospital/physician records (n=3), only self-reporting (n=2), national registries (n=1), and two studies did not report how they collected information on diabetes status. Importantly, two studies identified associations

between arsenic and gestational diabetes [25, 46], as opposed to the remainder of studies that examined adult onset DM. In addition to these studies of gestational diabetes, there has been an additional study that has shown that women that develop gestational diabetes have a higher level of U-tAs than their non-diabetic counterparts [59]. As before, these studies used a variety of adjustment factors, with age, sex and/or BMI being common (Supplemental Table 1). Additionally, these studies tended to consider the impact of race, creatinine, smoking status, and alcohol consumption more often than studies at higher exposure levels (Supplemental Table 1). Studies at the lower end of arsenic exposure tended to have mixed results, with significant attention being paid to adjustment factors [60-62]. For example, some studies which analyzed the same dataset and found opposing results depending on what covariates have been used [52, 53, 55, 58]. Creatinine has been shown to be higher in diabetics as compared to their non-diabetic counter parts, and thus adjustment of U-tAs would result in an overestimate of arsenic exposure in diabetic individuals [63]. While the association between arsenic and diabetes is much less clear at lower exposures, the majority (n=25, 73.5%) of these studies did identify an association.

At a lower level of exposure, studies have also looked at the impact of arsenic metabolism on the development of DM. Studies have shown that individuals with diabetes tend to have higher levels of U-tAs than their non-diabetic counterparts [59, 64-67]. Interestingly, both studies of blood arsenic levels have shown no difference between diabetic and non-diabetic individuals [67, 68]. This is likely because arsenic is cleared from blood within 4 hours, and thus represents a very immediate exposure to arsenic and not a chronic body burden. Additionally, it has been shown that individuals with diabetes have a different metabolomics profile than their non-diabetic counterparts exposed to arsenic, suggesting that numerous metabolic processes in addition to arsenic metabolism differ between these two groups [69]. These data clearly

represent an area of research that should be further explored. Additionally, as the only two prospective cohort studies, which could provide evidence for a causal link between arsenic and diabetes, have been conducted to date, there is a need for better study designs.

In pooling the conclusions from these studies, two meta-analyses have been determined there is an association between chronic arsenic exposure at levels < 150µg iAs/L and diabetes [70, 71]. These meta-analyses found pooled relative risks of developing diabetes in the presence of arsenic equal to 1.7 and 1.23, respectively [70, 71]. Additionally, some studies in arsenic-exposed human populations where arsenic is associated with biomarkers of pathological processes typically associated with DM. Specifically these biomarkers represent renal damage, oxidation stress, low-grade inflammation, and endothelial damage [72]. However, at present it is unclear what mechanisms are most relevant to the development of arsenic-associated diabetes in human populations. There is a gap in the knowledge regarding how arsenic's induction of diabetes occurs in human population. Elucidating these mechanisms could be key for the development of effective intervention strategies and clinical treatment of the disease.

iii. Mechanisms of arsenic-associated diabetes

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia that results from disruption of key processes involved in the regulation of glucose homeostasis. The sustained hyperglycemia is the result of the failure to produce or secret insulin in the pancreatic β-cells and/or the failure of peripheral tissues to respond to insulin signaling. Type 1 diabetes is the result of an autoimmune condition where pancreatic β-cells are destroyed resulting in a loss of insulin and the subsequent inability of tissues to utilize blood glucose. In contrast, type 2 diabetes is characterized by impaired insulin signaling in peripheral tissues, i.e.,

insulin resistance, and subsequent β-cell dysfunction. Both type 1 and 2 diabetes are characterized by hyperglycemia. Interestingly, it has been demonstrated that laboratory animals exposed to inorganic arsenic develop phenotypes consistent with DM, namely increased fasting blood glucose, impaired glucose tolerance and/or insulin resistance [73-79]. Additionally, prenatal exposure to inorganic arsenic predisposes offspring to the development of diabetes in laboratory studies [80, 81]. In eleven of the thirteen currently published rodent studies, the doses used to promote diabetes in laboratory animals were much higher than those typically associated with the environmental exposures. It is important to note that these higher doses may be necessary as mice are less susceptible to arsenic-induced health effects than humans due to more efficient metabolism and clearance [82].

In vitro and *in vivo* laboratory studies support four major mechanisms by which inorganic arsenic and its methylated metabolites are able to influence DM-related processes (Table 1). The first mechanism is the inhibition of insulin signaling, resulting in insulin resistance and in an impaired glucose uptake and utilization. The second mechanism is the induction of apoptosis of pancreatic β-cells, the primary site of insulin synthesis. The third mechanism is the inhibition of glucose stimulated insulin secretion by pancreatic β-cells at non-cytotoxic concentrations. The fourth mechanism is induction of gluconeogenesis. While these processes are also involved in type 2 diabetes [79] the ability of arsenic to dysregulate these processes suggests it is a diabetogen.

In addition to inorganic arsenic exposure, different methylated metabolites of arsenic influence processes involved in the development of diabetes to different degrees, suggesting that metabolism of arsenic is key to likelihood of diabetes development. While the majority of arsenic taken up into the body is inorganic arsenic, it is metabolized by the enzyme arsenic (+3-

oxidation state) methyltransferase (AS3MT) to different forms. AS3MT, and to a lesser extent other enzymes first methylate and reduce inorganic arsenic to produce monomethyl arsenicals ($MMAs^{3+}$, $MMAs^{5+}$). This process is repeated to produce dimethyl arsenicals ($DMAs^{3+}$, $DMAs^{5+}$). Importantly, it has been shown that the trivalent arsenicals are much more potent that the pentavalent counterparts as inhibitors of insulin signaling and insulin secretion by β-cells, suggesting that the trivalent species may be the main drivers of arsenic-associated diabetes [83]. However, it is important to note that limited evidence on pentavalent arsenical species hinders the ability to understand their roles in the induction of arsenic-associated DM. The use of biomarkers of cell injury and altered glucose signaling provides support that arsenic-induced injuries resulting in diabetes occur in humans. Taken together, the following studies of the effect of arsenic on glucose homeostasis pathways provide cohesive evidence that arsenic can cause the development of DM.

Mechanism 1: arsenic inhibits insulin-dependent glucose uptake
• Inhibition of GLUT4 localization to the plasma membrane
• Inhibition of Akt signaling to mobilize GLUT4
• Alterations of gene expression associated with Akt pathway
• Upregulation of antioxidant pathways leading to decreased secondary messengers
• Influence development of glucose transport system
Mechanism 2: β-cell damage
• Induction of apoptosis due to production of ROS
• Induction of chronic inflammation due to production of ROS
Mechanism 3: β-cell dysfunction
• Failure to secrete insulin specifically in response to glucose in response to ROS
Mechanism 4: stimulation of gluconeogenesis
• Induces expression of phosphoenolpyruvate carboxykinase

Table 1. Summary of major mechanisms underlying arsenic-associated diabetes

a. Mechanisms of arsenic-associated diabetes mellitus: inhibition of insulin-dependent glucose uptake (insulin resistance)

Arsenic and its metabolites can alter glucose homeostasis by causing insulin resistance and impaired glucose uptake [82-84]. Arsenite (iAs^{3+}), and its trivalent metabolites, methylarsonous acid ($MMAs^{3+}$), and dimethylarsonous acid ($DMAs^{3+}$), inhibits glucose transporter type 4 (GLUT4) recruitment to the plasma membrane despite insulin stimulation *in vitro* [83] (Figure 1). Further, *in vitro* studies have demonstrated that iAs^{3+} and $MMAs^{3+}$ inhibit the insulin-dependent activation of protein kinase B (Akt), a key signaling step required for GLUT4 translocation to the plasma membrane, by preventing its phosphorylation by PDK-1 [77, 85] (Figure 1). $DMAs^{3+}$ works downstream of Akt to prevent the recruitment of GLUT4, but the exact mechanism remains unclear (Figure 1). The process of Akt inhibition is compounded by the fact that arsenic can influence the expression of the PI3K transcription factor that is upstream of Atk, and that regulates other important steps in insulin signaling *in vivo* and *in vitro* [85, 86].

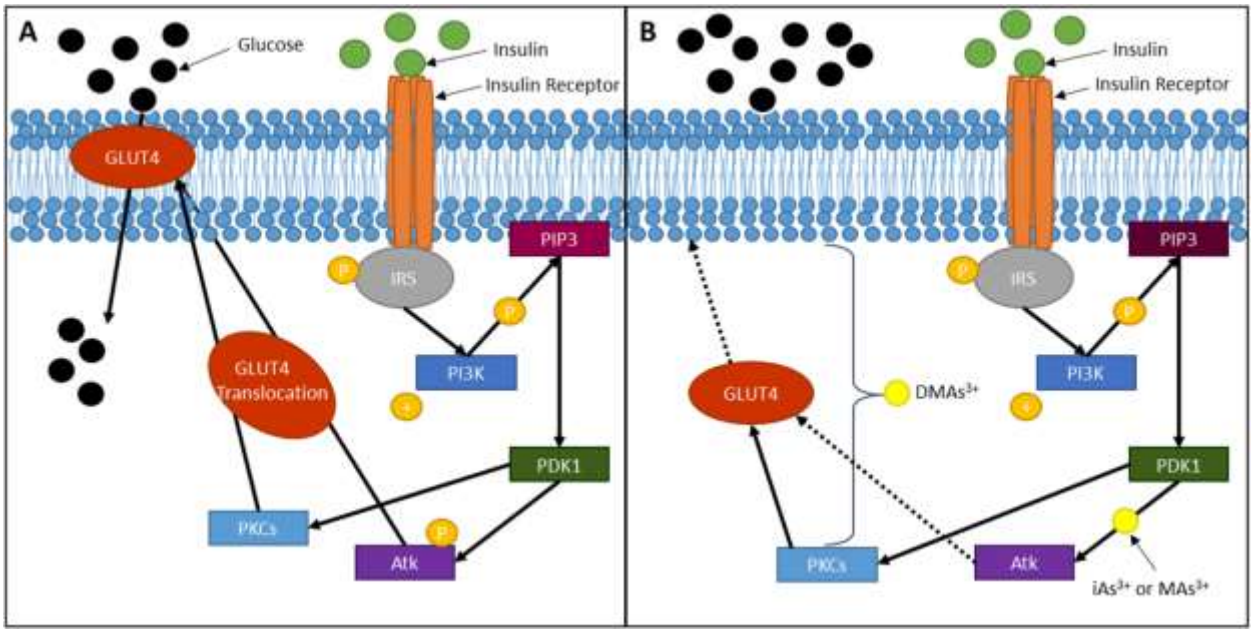


Figure 1. Depiction of Mechanism 1 of arsenic associated diabetes. iAs^{3+} and its trivalent metabolites inhibit insulin-dependent glucose uptake by disrupting the insulin-activated signaling

cascade. iAs^{3+} and $MMAs^{3+}$ have been shown to inhibit the phosphorylation of Akt by PDK, resulting in inhibition of GLUT4 translocation to plasma membrane; $DMAs^{3+}$ inhibits signaling downstream of PDK/Akt, but the exact target has not been identified. Figure adapted from Paul et al. 2007 [77].

Additionally, the expression of many of the genes in this pathway, including *GLUT4*, and *Akt* are decreased in response to arsenic exposure *in vivo* and *in vitro* [84, 86, 87]. In mice, arsenic exposure is associated with generation of reactive oxygen species (ROS) and upregulation of cellular antioxidant pathways including Nuclear factor erythroid 2-related factor 2 (Nrf2) [88, 89]. The constitutive upregulation of Nrf2 and the consequent increase in antioxidant enzyme expression could inhibit insulin-stimulated glucose uptake as the insulin pathway may also require ROS as signaling molecules [87].

In addition to directly inhibiting the process of insulin-dependent glucose uptake, arsenic can interfere with adipogenesis, the differentiation of pre-adipocytes to adipocytes, as well as differentiation of myoblast into myotubes in skeletal muscle at doses between 0.5 and 5 μM [84, 90, 91]. Adipocytes in white adipose tissue and myotubes in skeletal muscle tissue play critical roles in glucose utilization and in the maintenance of blood glucose levels. Arsenic inhibits adipocyte differentiation by altering the expression of key genes and transcription factors involved in adipogenesis, specifically peroxisome proliferator-activated receptor γ (*PPAR-γ*), and CCAAT/enhancer binding protein α (*C/EBPα*) [85, 90, 92, 93]. This indirectly affects numerous other proteins involved in adipocyte differentiation including p21 and A-FABP [94]. Alterations of adipogenesis likely lead to decreased lipid storage capacity and insulin resistance, as it has been shown that defects in adipogenesis are can result in insulin impairment and type two diabetes [90, 95].

It is also possible that arsenic influences the development of the insulin responsive glucose transport system [84], by decreasing the phosphorylation of mechanistic target of rapamycin (mTOR) and ribosomal protein S6 kinase B1 (p70), key regulators in this process [96]. Notably, in humans, urinary total arsenic (U-tAs) (a biomarker measure of arsenic exposure) is inversely associated with insulin sensitivity levels [97]. However, it has also been shown that arsenic exposure is inversely related to fasting plasma insulin and to the measure of insulin resistance (HOMA-IR) [36]. Similarly, mice that were exposed to arsenic and fed a high fat diet displayed impaired glucose tolerance and a dose-dependent decrease in HOMA-IR, likely due to lower fasting insulin and higher fasting blood glucose than in mice fed a low fat diet [79]. Insulin response to glucose challenge was also impaired in these mice. Thus, both impaired insulin sensitivity and impaired insulin secretion in response to high blood glucose may underlie the diabetogenic effects of arsenic exposure.

b. Mechanisms of arsenic-associated diabetes mellitus: β-cell damage

Arsenic can damage pancreatic β-cells. It has been shown that arsenic metabolites accumulate in the pancreases of mice exposed to arsenic [73, 78, 82]. Arsenic accumulation induces apoptosis of pancreatic β-cells *in vitro,* likely through the production of ROS, and induction of PARP and Caspase-3, as well as through other mechanisms [91, 98-100]. In addition to causing cell death, this accumulation of ROS has been shown to result in chronic inflammation. Such chronic inflammation can result in pancreatitis *in vivo* which can lead to the development of diabetes [100]. Moreover, excessive levels of ROS result in upregulation of antioxidant pathways. It has been proposed that a persistent activation of Nuclear factor (erythroid-derived 2)-like 2(Nrf2), a key transcription factor in the antioxidant system, reduces glucose-triggered ROS-mediated signaling and thus inhibits glucose-stimulated insulin secretion

in vitro [101]. Exposure to As^{3+} and $MMAs^{3+}$ have also been shown to lead to inhibition of thioredoxin reductase *in vivo*, a key member of the cellular anti-oxidant defense, thus resulting in apoptosis [73, 102].

c. Mechanisms of arsenic-associated diabetes mellitus: β-cell dysfunction

Arsenic and its methylated metabolites can inhibit insulin gene transcription and/or secretion in the pancreas. Some studies suggested that iAs^{3+} may decrease the levels of insulin mRNA produced by pancreatic β-cells. Decreased insulin mRNA production would produce the same effect as destruction of these cells, however there have been conflicting data on the subject, likely due to dose differences [103, 104]. The destruction of cells by ROS-mediated mechanisms, as well as other cellular targets of iAs^{3+}, directly impacts the ability of the pancreases to produce insulin. Recent *in vitro* research has demonstrated that exposure of pancreatic islets to low arsenic concentrations can produce insulin, but fail to secrete it results in a blunted glucose-stimulated insulin secretion [103, 104]. Consistent with these findings are results of a recent population study that showed arsenic exposure to be associated with decrease in the insulin secretion index, HOMA2%B [41]. An additional *in vitro* study using a β-cell line suggested that this is likely due to As^{3+} ability to inhibit calcium oscillation which is necessary for glucose stimulated insulin secretion [105]. Interestingly, *in vitro* both $MMAs^{3+}$ and $DMAs^{3+}$ are more potent inhibitors of glucose-stimulated insulin secretion by pancreatic islets than iAs^{3+}, though the mechanisms for this are unclear [104]. It has also been shown *in vitro* that the cellular adaptation to chronic oxidative stress caused by iAs^{3+} exposure lowers ROS production and impairs glucose-stimulated insulin secretion, which may require low level-ROS as one of the regulatory mechanisms [106].

d. Mechanisms of arsenic-associated diabetes mellitus: stimulation of gluconeogenesis

In addition to inhibiting insulin secretion, gene expression changes induced by arsenic *in vivo* can also induce hepatic gluconeogenesis. Hepatic gluconeogenesis is the pathway for synthesis of glucose from non-carbohydrate sources during fasting. Arsenic induces increased expression of phosphoenolpyruvate carboxykinase, a rate limiting enzyme in gluconeogenesis resulting in fasting hyperglycemia [107, 108]. In summary, these data provide potential mechanistic understanding of how arsenic is able to regulate glucose hemostasis resulting in hyperglycemia and DM.

iv. Genetic underpinnings for arsenic-associated diabetes mellitus

Inter-individual genetic differences have been associated with differential risk for arsenic-associated DM. Multiple studies have found that differences in the metabolism of inorganic arsenic and in the distribution and excretion of arsenic metabolites are associated with likelihood of diabetes development [37, 50, 109-111]. As detailed previously, inorganic arsenic (iAs) is methylated primarily by *AS3MT* to form the monomethylated and dimethylated arsenic metabolites, MMAs and DMAs. Studies of arsenic-exposed populations have found that higher iAs and MMAs in urothelial cells and higher DMAs in urine, are more strongly associated with diabetes than drinking water measures of arsenic or urinary total arsenic [35, 36, 50, 66]. These differences in arsenic metabolite profiles represent a potential indicator of the risk of developing diabetes and could be mediated by differences in genotypes, specifically single nucleotide polymorphisms (SNPs), of critical genes such as: *AS3MT* [112], Calpain 10 (*CAPN-10*) – a calcium-dependent protease that plays a key role in exocytosis of insulin containing vesicles in βcells [113], Glutathione S-Transferase Omega 1 (*GSTO1*)- a enzyme that contributes to the

reduction of arsenic from the pentavalent to trivalent form [110], and Notch 2 (*NOTCH2*) a member of a signaling cascade involved in cell differentiation [110, 111, 113, 114]. Interestingly, the majority of SNPs associated with differences in arsenic metabolism occur in non-coding regions of the genes, suggesting potential epigenetic regulation of gene expression or splice variants.

v. Metabolomics

Metabolomics is an untargeted screening of small molecule metabolites (i.e. products and substrates of metabolism). This can occur at the level of the cell, tissue, biological fluid or organism at a given point in time. The goal is to measure and identify as many metabolites as possible to understand the present of state of the biological system. Typically, mass spectrometry and NMR spectroscopy are the most comment techniques for metabolome profiling.

Metabolomics can be applied to assess the relationship between metabolite levels and various stimuli to inform what key processes are being altered in a biological response. This technology has numerous applications including understanding alterations associated with chronic disease state, environmental exposure, drug development, or infection. Metabolomics has been applied to assess diabetes with a total of 22 targeted and untargeted metabolomics studies assessing metabolomics impacts of diabetes[115]. This work represents one of the first of its kind as it was among the first to assess metabolomic impacts of arsenic exposure in humans. Specifically, the 22 studies of metabolomics profiles related to diabetes have found increased levels of the plasma branch chain and aromatic amino acids, specifically isoleucine, leucine, valine, tyrosine and phenylalanine are associated with development of diabetes. These studies have found alterations in sugars (glucose, fructose, and inositol) and lipids (phospholipids,

sphingomyelins, triglycerides) are associated with diabetes as well as are predictive markers of diabetes development.

vi. Project Approach

This research focuses on the use of metabolomics profiling to understand the response of diabetic and non-diabetic individuals to arsenic exposure. **The central hypothesis of this research is arsenic-induced diabetes is different from traditional type 2 diabetes and that metabolomics profiles of arsenic exposed diabetics and non-diabetics can provide key insights into disease development risk and disease profile.** This project focuses on characterization of metabolomics profiles in response to arsenic exposure, measures of arsenic metabolism and genetic inter-individual differences related to arsenic metabolism. We assess metabolomics profiles across two matrices, urine and plasma, to understand the key metabolic pathways perturbed by arsenic that potentially lead to diabetes. It is notable that our research is the first to (1) assess metabolomics profiles in response to arsenic exposure in nominally healthy individuals (2) integrate metabolomics in an exposure X disease paradigm and (3) assess the relationship between metabolomics profiles and inter-individual differences related to toxicant metabolism and genetic variation.

vii. Dissertation Organization

This dissertation is organized into three chapters. The first chapter describes the characterization of metabolites associated with urinary total arsenic in diabetic and non-diabetic individuals. The second chapter expands upon this to examine the role of arsenic metabolism on metabolite profiles in diabetic and non-diabetic individuals. The third chapter examines the relationship between genetic variation and metabolite profiles to understand genetic

susceptibility to arsenic-associated disease in a human population. Of particular interest these studies identify key pathway alterations unique to diabetic individuals exposed to arsenic. Additionally, we identify alterations in metabolites that have been previously associated with likelihood of development of diabetes. Finally, metabolic alterations associated with genotypic differences provide insight into differences related to inter-individual susceptibility to arsenic-associated diabetes. These three studies provide insight into susceptibility and development of arsenic-associated diabetes.

CHAPTER 1: METABOLOMIC CHARACTERISTICS OF ARSENIC-ASSOCIATED DIABETES IN A PROSPECTIVE COHORT IN CHIHUAHUA, MEXICO

1.1 Overview

Drinking water contaminated with inorganic arsenic (iAs) is a major threat to human health with more than 100 million people worldwide exposed to levels that exceed the World Health Organization's (WHO) recommended limit of 10 μg As/L [116]. Chronic exposure to arsenic has been linked to an increased risk of cancer and non-cancerous diseases, including cardiovascular disease and diabetes mellitus (DM) [117-121]. *In vivo* and *in vitro* studies arsenite (iAs^{3+}) and its trivalent methylated metabolites inhibit insulin signaling, insulin-dependent glucose uptake, insulin secretion, and induce apoptosis in pancreatic β-cells [122]. Understanding which of these mechanisms contribute to the phenotype of arsenic-associated diabetes and mechanisms underlying this disease is essential for the development of effective treatment strategies in areas where drinking water is contaminated with arsenic.

Given that metabolomics assessment allows for untargeted screening of a vast number of metabolites and allows for discovery of key pathways related to disease state and exposure we set out to utilize it in understanding the development of arsenic-associated diabetes. In the present work we chose to focus on urine and plasma metabolomics profiles as these represent easily obtainable bio-specimens. We measured 429 unique metabolites, 221 in plasma and 294 in urine, using LC-MS-TOF/GC-MS-TOF in 90 diabetic and 86 non-diabetic individuals. These

individuals came from a recently established a cohort of 1,165 Chihuahua residents exposed to a wide range of arsenic concentrations.

A total of 132 metabolites were identified to shift in urine or plasma in response to arsenic exposure characterized by the sum of arsenic metabolites in urine (U-tAs). While 33 metabolites were altered in both diabetic and non-diabetic subjects, diabetic individuals displayed a unique response to arsenic exposure with 59 altered metabolites. These metabolites were also different from metabolites associated with type 2 diabetes in previous research. The metabolites uniquely altered in response to arsenic exposure in diabetic individuals displayed enrichment of metabolic processes related to tricarboxylic acid cycle and amino acid metabolism.

1.2 Study Objectives

For this study, we set out to assess whether metabolomics profiles of arsenic-exposed non-diabetic and-diabetic individuals were different. We tested this by comparing the metabolomics profiles of arsenic-exposed diabetic and non-diabetic individuals. Using Liquid Chromatography/Gas Chromatography coupled to Mass Spectroscopy, we assessed 426 unique urine and plasma metabolites. Using multivariable linear regression, we determined arsenic-associated metabolites in diabetic and non-diabetic individuals. We then used pathway analysis to examine the context related to the changes we observed in metabolite alterations. Taken together this research suggests that diabetics exposed to arsenic develop a phenotype different from traditional type 2 diabetes.

1.3 Materials and Methods

1.3.1 Study population

All procedures involving human subjects were approved by the IRBs of UNC Chapel Hill and Cinvestav-IPN, and all participants signed a written consent. A total of 1,165 adults (≥18 years old) with a minimum of 5-year uninterrupted residency in Chihuahua, Mexico were recruited between 2008 and 2012 [123, 124]. Pregnant women and subjects reporting kidney or urinary tract infection were excluded because these conditions may affect profiles of arsenic metabolites in urine. Individuals with potential occupational exposure to arsenic (e.g., those working with pesticides or in mines or smelters) were also excluded. Samples of drinking water were obtained from each subjects' households. An interviewer-administered study questionnaire was used to record data on residency, occupation, drinking water sources and use, smoking, alcohol consumption, and medical history. Spot urine and fasting venous blood were collected during medical exams which included an oral glucose tolerance test with blood drawn 2 hours after a 75 g glucose dose. Plasma from both fasting and two-hour blood samples and urines were immediately frozen and stored at -80^{o}C. Measures of body weight and height were obtained during the exams and used to calculate body mass index (BMI).

The metabolomics analyses focused on a select set matched set of 90 diabetic and 90 non-diabetic individuals. For comparison, 90 non-diabetic individuals were randomly selected from the cohort within strata of arsenic concentration in drinking water (<10, 10-49, 50-149, ≥150 μg iAs/L), BMI (<25, 25-29, ≥30), sex and age (≤39, 40-49, 50-59, ≥60) to match the distribution of these factors among the cases. The matching criteria were relaxed in four instances when there were no non-diabetic individuals in the same stratum as the case. In addition, four non-diabetic subjects were subsequently excluded from the study because of

incorrect or missing data on arsenic in water, BMI or use of diabetic medication. Thus, the final numbers of subjects included in the nested sub-cohort were 90 diabetic and 86 non-diabetic individuals.

1.3.2 Diabetes determination

Individuals were classified as diabetic if they had a fasting plasma glucose (FPG) ≥126 mg/dL or two-hour blood glucose (2HPG) ≥200 mg/dL. Individuals who reported previous diabetes diagnosis or use of diabetic medication but with both FPG <126 mg/dL and 2HPG <200 mg/dL were not treated as diabetic individuals. Blood glucose levels, both fasting plasma glucose (FPG) and 2-hour plasma glucose (2HPG), were measured using a Prestige 24i Chemistry Analyzer (Tokyo Boeki, Tokyo, Japan). The analyzer was calibrated to ensure accuracy prior to analysis with human sera containing both normal and elevated glucose levels (Serodos and Serodos PLUS, Human Diagnostics Worldwide).

1.3.3 Arsenic assessment

Hydride generation-atomic absorption spectrometry coupled with a cryotrap (HG-CT-AAS) was used to determine the concentration of concentrations of arsenic species in urine: total iAs, total monomethyl-As (MMAs) and total dimethyl-As (DMAs)[125]. Metabolites were then summed together for the measure of urinary total arsenic (U-tAs). To ensure accuracy a certified standard reference material Arsenic Species in Frozen Human Urine (SRM 2669; National Institute of Standards and Technology) was analyzed with every single batch of urine samples shipped from the field to UNC. The concentrations of iAs species measured by HG-CT-AAS in SRM 2669 ranged from 86.7 to 106.4% of the certified values. The limit of detection (LOD) for

arsenic in water was 0.1 µg iAs/L; LODs for iAs species in urine were 0.05 ng As/mL for MMAs or DMAs and 0.1 ng iAs/mL for iAs.

1.3.4 Metabolomics assessment

Metabolic profiling was carried out in plasma and urine using gas chromatography (GC) and liquid chromatography (LC) with time of flight-mass spectrometry (TOF-MS) detection [126-129]. Here, the GC-TOF-MS were obtained with electron impact ionization (70 eV) at full scan mode (m/z 40-600) with a Rxi-5ms capillary column (30 m × 250 µm i.d., 0.25-µm film thickness, Restek, PA, USA) on a Pegasus HT system (Leco Co., St Joseph, MI) coupled with an Agilent 6890 GC (Agilent Co., Santa Clara, CA), using helium as the carrier gas at a constant flow rate of 1.0 mL/min. The acquired data files were analyzed by ChromaTOF software (Leco Co.). The LC-TOF-MS mass spectra were obtained with Thean Agilent HPLC 1200 system coupled with 6220 MSD TOF-mass spectrometer (MS) (Agilent CorporationCo., Santa Clara, CA). The acquired GC-TOF-MS and LC-TOF-MS data were processed using ChromaTOF software (Leco Co., St Joseph, MI), Agilent MassHunter Qualitative Analysis Program (vB.05.00), and XCMS package (v1.24.1, http://metlin.scripps.edu) [130, 131]. Spectral data conversion was performed using the Agilent MassHunter Qualitative Analysis Program (vB.05.00) and XCMS package. Metabolites were verified and annotated using an in-house library of >800 mammalian metabolites and the on-line databases, including the Human Metabolome Database (http://www.hmdb.ca/), the National Institute of Standards and Technology library, and the LECO/Fiehn Metabolomics Library. Relative amounts of the metabolites (individual metabolite peak area divided by the sum of all metabolite peak areas) were used in statistical analyses.

1.3.5 Statistical Analyses

Data were analyzed using the statistical packages SAS 9.3 (SAS Institute Inc., Cary, NC) and Partek® Genomics Suite TM Software (St. Louis, MO). A Spearman rank test was used to quantify the relationship between DW-iAs and U-tAs. Descriptive statistics were used to summarize iAs in drinking water, U-tAs, and other demographic factors. Because specific gravity was found to be associated with diabetes status within this cohort [132] and urinary creatinine levels have been previously linked with arsenic exposure, unadjusted values of U-tAs were used in the primary model in the analyses [63, 133, 134]. The abundance levels of the 221 plasma metabolites and 294 urine metabolites were used for analyses. Human Metabolome Database Identifiers (HMDB IDs) were also determined for metabolites to facilitate comparison between other studies.

The GC-TOF-MS and LC-TOF-MS analyses identified 294 urinary and 221 plasma metabolites, including 89 metabolites that were detected in both plasma and urine with a total of 426 unique metabolites that were profiled in this study (Supp Table 1, Supp Table 2). Multi-variable regression models were used to establish relationships between metabolite levels and arsenic exposure in diabetic and non-diabetic individuals, where U-tAs was used as the main predictor variable and metabolite levels the dependent variable. BMI, age and sex, were selected as covariates based on *a priori* association with diabetes. Statistical significance was set at $p<0.05$, $q<0.2$.

1.3.6 Enrichment Analyses

Associations of statistically significantly metabolites with canonical metabolic pathways were determined using the MetPA tool within Metabolomics Pathway Analysis [135]. This tool

maps metabolites onto KEGG pathways and determines the statistical significance as well as the impact of metabolite groups. Human Metabolome Database (HMDB) identifiers were used for these analyses as they provided greater coverage of metabolites than did the metabolite names [135]. As the metabolites had already been statistically filtered, a more relaxed statistical significance of $p<0.1$ was used in the pathways analysis.

1.4 Results

1.4.1 Demographic description of the sub-cohort

The sub-cohort selected for the present study (n = 176) consisted of 133 (75.6%) women and 43 (24.4%) men (Table 2). Men and women were equally represented in the diabetic and non-diabetic groups (Table 2). There were no statistically significant differences on average between the two groups for BMI, age, drinking water iAs or U-tAs (Table 2). Among the 176 individuals, a total of 152 (86.9%) individuals were exposed to DW-iAs levels exceeding 25 µg iAs/L, the current maximum allowable contaminant value for iAs for drinking water supplies in Mexico.

	All Individuals (n=176)		Non-Diabetic Individuals (n=86)		Diabetic Individuals (n=90)	
A. Demographics	**N (%)**	**Mean (range)**	**N (%)**	**Mean (range)**	**N (%)**	**Mean (range)**
Sex						
Female	133 (75.6%)	-	65 (75.6%)	-	68 (75.6%)	-
Male	43 (24.4%)	-	21 (24.4%)	-	22 (24.4%)	-
Age (years)	-	50 (18-79)	-	50 (18-78)	-	51 (23-79)
BMI	-	30.4 (18.3-45.2)	-	30.1 (19.2-43.6)	-	30.7 (18.3-45.2)
Fasting Blood Glucose	-	131.1 (49.5-379.0)	-	88.7 (49.0.5-117)*	-	169.7 (63.0-379.0)*
2-Hour Blood Glucose	-	163.8 (50.0-437.0)	-	112.5 (50.0-191.5)*	-	211.1 (50.5-437.0)*
B. Arsenic Exposure	**N (%)**	**Mean (range)**	**N (%)**	**Mean (range)**	**N (%)**	**Mean (range)**
DW-iAs (µg/L)	-	68.4 (LOD-292.9)		65.8 (LOD-284.7)		70.8 (LOD-284.7)
≤ 10 µg/L	20 (11.3%)	-	11 (12.8%)	-	9 (10.0%)	-
≥ 10 µg/L	156 (88.7%)	-	75 (87.2%)	-	81 (90.0%)	-
≤ 25 µg/L	24 (13.1%)	-	14 (16.3%)	-	10 (11.1%)	-
≥ 25 µg/L	152 (86.9%)	-	72 (83.7%)	-	80 (89.9%)	-
U-tAs[1]	-	86.8 (3.85-348.6)	-	80.5 (6.11-348.61)	-	92.6 (3.85-346.3)

Table 2. Demographic Characteristics of the Cohort that was metabolically profiled.

Only 20 water samples (11.3%) had DW-iAs below the WHO recommended value of 10 µg iAs/L. DW-iAs was significantly correlated with U-tAs: r = 0.37 p<0.01 (Figure 2). The key characteristics of the entire Chihuahua cohort were described elsewhere [124, 132].

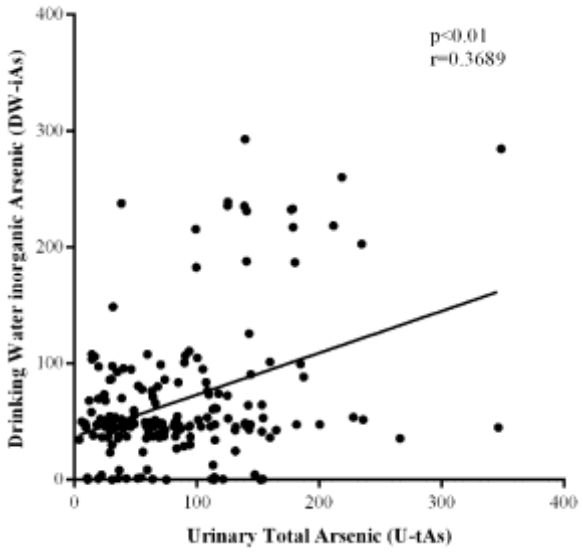


Figure 2. Correlation between urinary total arsenic (U-tAs) and drinking water arsenic (DW-iAs) that demonstrates a correlation between the two measures of exposure.

1.4.2 Characteristics of the metabolome

Of the 426 unique metabolites measured, a total of 132 metabolites were significantly increased or decreased with exposure to arsenic (characterized by U-tAs) among either diabetic or non-diabetic individuals (Supplemental Table 2). Of these, 103 were urinary and 32 were plasma metabolites (Table 3), with three metabolites common to both urine and plasma (Supplemental Table 2). However, the response to arsenic exposure differed between the matrices. While in urine the concentrations for most of the altered metabolites (n=68, 66%)

increased, the concentrations of most of the plasma metabolites (n=20, 63%) decreased (Table 3, Supplemental Table 2).

	Urine n (%)	Plasma n (%)	Overall n
Overall	**103**	**32**	**132**
Increased Metabolite Level	68 (66%)	9 (31%)	77
Decreased Metabolite Level	35 (34%)	20 (68%)	55
Non-diabetic Individuals	**61**	**15**	**73**
Increased Metabolite Level	35 (57%)	3 (20%)	36
Decreased Metabolite Level	26 (43%)	12 (80%)	37
Diabetic Individuals	**73**	**17**	**87**
Increased Metabolite Level	52 (71%)	9 (53%)	59
Decreased Metabolite Level	21 (29%)	8 (47%)	28

Table 3. Number of U-tAs associated-metabolites in either plasma or urine.

1.4.3 Metabolites associated with arsenic exposure in urine and plasma

Of the 426 unique metabolites measured, a total of 132 metabolites were significantly increased or decreased with exposure to arsenic (characterized by U-tAs) among either diabetic or non-diabetic individuals (Supplemental Table 2). Of these, 103 were urinary and 32 were plasma metabolites (Table 3), with three metabolites common to both urine and plasma (Supplemental Table 2). However, the response to arsenic exposure differed between the matrices. While in urine the concentrations for most of the altered metabolites (n=68, 66%) increased, the concentrations of most of the plasma metabolites (n=20, 63%) decreased (Table 3, Supplemental Table 2).

In urine, a total of 61 metabolites were associated with U-tAs among non-diabetic subjects and 73 metabolites were associated with U-tAs among diabetic subjects (Figure 3a, Table 3, Supplemental Table 2). Of those metabolites, 30 were unique to non-diabetic individuals, 42 were unique to diabetic individuals, and 31 metabolites were associated with U-

tAs in both groups (Figure 3a). Among the 31 common metabolites were those related to amino acid metabolism, the tricarboxylic acid (TCA) cycle, and pyruvate metabolism. The majority of the urinary metabolites showed a positive association with arsenic exposure: 35 of the 61 metabolites in non-diabetics, and 52 of the 73 metabolites in diabetic individuals (Table 3, Supplemental Table 2).

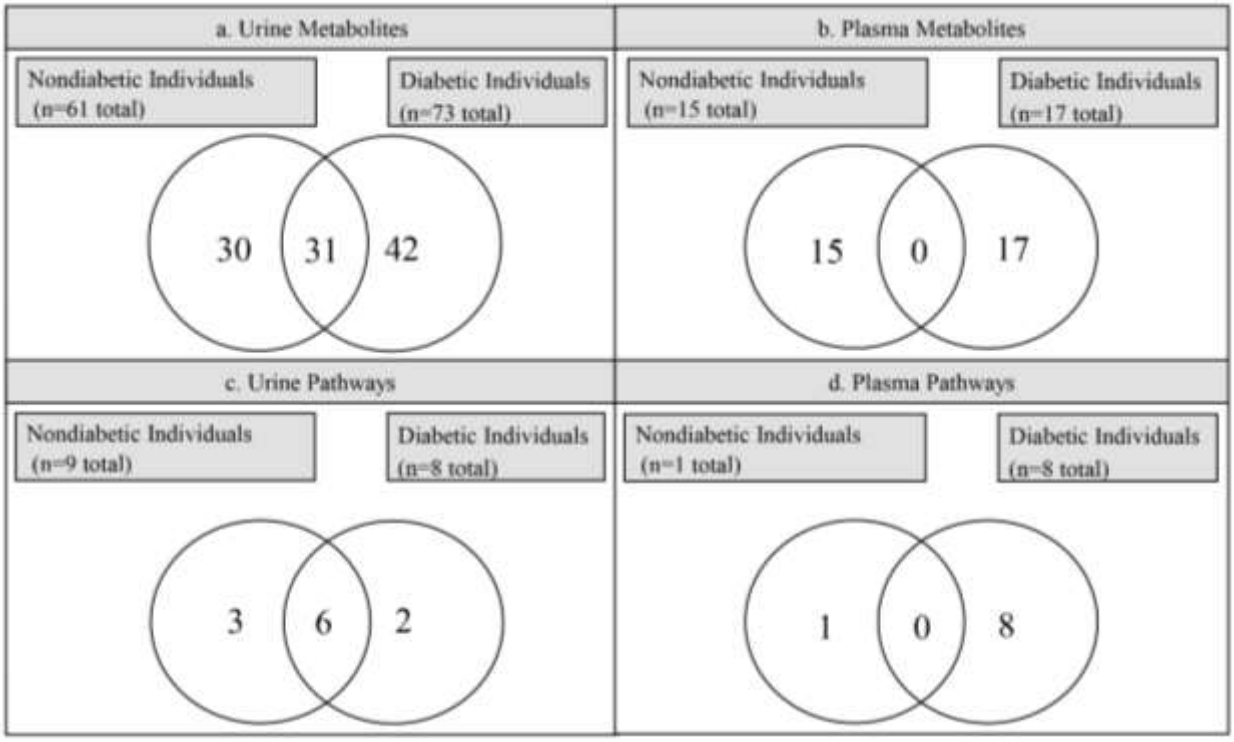


Figure 3. Comparison of plasma and urinary metabolites and pathways associated with arsenic exposure in diabetic and non-diabetic individuals.

In plasma, a total of 15 metabolites were associated with U-tAs among non-diabetic individuals and 17 metabolites were associated with U-tAs among diabetic individuals (Figure 3b, Supplemental Table 2). No U-tAs-associated metabolites in plasma were common among the diabetic and non-diabetic subjects (Figure 3b, Supplemental Table 2). Many of the plasma metabolites were negatively associated with arsenic exposure: 8 of the 17 metabolites in

diabetics and 12 of the 15 metabolites in non-diabetic individuals (Table 3, Supplemental Table 2). Thus, across the two matrices diabetic individuals displayed altered levels of 59 metabolites (42 urinary metabolites and 17 plasma metabolites) (Supplemental Table 2).

1.4.4 Identification of arsenic-associate diabetes finger print

Using METPA to perform KEGG enrichment analysis, the 61 urinary metabolites associated with U-tAs in non-diabetic individuals were enriched for nine metabolic pathways (Figure 3c, Table 4). Three of these pathways were associated with amino acid metabolism (alanine/aspartate/glutamate, phenylalanine, arginine and proline), five with carbohydrate/energy metabolism (TCA cycle, glyoxylate/dicarboxylate, galactose, pentose/glucoronate, and pyruvate), and one was associated with vitamin (riboflavin) metabolism (Table 4).

The 73 urinary metabolites identified in diabetic individuals enriched for eight pathways (Figure 3c, Table 4). Of these pathways, three were associated with amino acid metabolism (alanine/aspartate/glutamate, phenylalanine, taurine) and four with carbohydrate/energy metabolism (TCA cycle, glyoxylate, pentose and pyruvate). One pathway was associated with nucleic acid (purine) metabolism (Table 4).

When examining the overlap between the study groups at a pathway level, there were six pathways that were associated with U-tAs in both non-diabetic and diabetic subjects. These were pathways for metabolism of alanine/aspartate/glutamate, glyoxylate/dicarboxylate, pentose/glucoronate, phenylalanine, and pyruvate, and TCA cycle (Figure 3c, Table 4). Three pathways were unique to the metabolome of non-diabetic subjects, including the pathways of arginine/proline, galactose, and riboflavin metabolism. Two pathways involved in purine and taurine metabolism were unique to the metabolome of diabetic subjects.

When KEGG enrichment analysis was conducted for the 15 plasma metabolites associated with U-tAs in non-diabetic individuals, a single pathway was enriched, namely the pathway for biosynthesis of phenylalanine, tyrosine and tryptophan (Figure 3d, Table 4). The 17 plasma metabolites associated with U-tAs in diabetic individuals enriched for eight pathways (Figure 3d, Table 4). Of these eight, five are associated with amino acid metabolism (arginine/proline, lysine, phenylalanine, and tyrosine and aminoacyl-tRNA biosynthesis), two are associated with energy metabolism (nitrogen metabolism and TCA cycle), and one pathway is associated with thiamine metabolism (Table 4). An examination of the overlap between the study groups at a pathway level revealed that there were no pathways associated with U-tAs in both non-diabetic and diabetic subjects (Figure 1d). Specifically, there was one pathway unique to the response of non-diabetic subjects to arsenic exposure, and eight pathways unique to the diabetic response to arsenic exposure.

As a separate analysis, the 59 metabolites that were uniquely associated with U-tAs in diabetic individuals (i.e. 42 urinary metabolites and 17 plasma metabolites) were also assessed for their enriched metabolic pathways, a total of 10 metabolic pathways were identified (Table 4). Among these, were 5 pathways related to amino acid metabolism, 3 related to energy metabolism and 2 related to B vitamin (biotin, riboflavin, thiamine) metabolism.

Class of Metabolism	Metabolic Pathway	Urine	Urine	Plasma	Plasma	Urine and Plasma
		Non-Diabetic Individuals (n=61)	**Diabetic Individuals (n=73)**	**Non-Diabetic Individuals (n=15)**	**Diabetic Individuals (n=17)**	**Diabetes Unique metabolites (n=59)**
Amino acid metabolism	Alanine, aspartate and glutamate metabolism	**p=0.001** Oxaloacetate (-), N-Acetyl-L-aspartate(+), Aspartate (-), Glutamine (-)	**p=0.001** N-Acetyl-L-aspartate (+), Aspartate (-), Succinate(+)			**p=0.053** Furmurate (+, P), succinate (+,U)
Amino acid metabolism	Aminoacyl-tRNA biosynthesis				**p<0.001** Glycine (+), Lysine (-), Tyrosine(-), Proline(+)	**p<0.001** Glycine (+, P), Serine (+, U), Methionine(+, U), Lysine (-, P), Proline (+, P)
Amino acid metabolism	Arginine and proline metabolism	**p=0.061** Glutamine (-), Guanidinoacetate (-), Creatinine (+), Pyrrole-2-carboxylate (-)			**p=0.004** Fumarate (+), Citrulline (-), Proline (+)	
Amino acid metabolism	Glycine, Serine, Threonine Metabolism					**p=0.039** Serine(+, U), Glycine (+, P), 5-Aminolevulinic acid(-, U)
Amino acid metabolism	Lysine Degradation				**p=0.01835** Glycine (+), Lysine (-)	**p=0.006** Glutaric acid (+, U), Lysine (-, P), glycine (+, P), Carnitine (-, U)
Amino acid metabolism	Phenylalanine, tyrosine and tryptophan biosynthesis			**p=0.086414** Erythrose 4-phosphate (-)		
Amino acid metabolism	Phenylalanine metabolism	**p=0.056** Pheynlpurvate (-), Acetamide (-), 4-Hydrobenzoate (-)	**p=0.010** Phenylpyruvate (-), Acetamide (-), Succinate (+)		**p=0.017** Tyrosine (-), Fumarate (+)	**p=0.005** Tyrosine (-, P), Phenylglyoxylic acid (+, U), Fumarate (+, P), succinate (+, P)
Amino Acid Metabolism	Taurine and hypotaurine metabolism		**p=0.055** Acetylphosphate (-), Taurine (-)			
Amino acid metabolism	Tyrosine Metabolism				**p=0.045** Tyrosine (-), Fumarate (+)	
Energy metabolism	Citrate cycle (TCA cycle)	**p<0.001** Malate (+), Oxaloacetate (-), Aconitate (+), Isocitrate (+)	**p=0.006** Malate (+), Succinate (+), Aconitate (+)		**p=0.003** Malate (+), Fumarate(+)	**P=0.039** Furmarate (+, P), Succinate (+, U)
Energy metabolism	Galactose metabolism	**p=0.044** Glyceraldehyde 3-phosphate (-), Galactitol (+), Sucrose (+)				
Energy metabolism	Glyoxylate and dicarboxylate metabolism	**p=0.015** Oxaloacetate (-), Aconitate (+), Malate (+), Isocitrate(+)	**p=0.014** Oxalate (-), Malate (+), Aconitate (+), Succinate (+)			
Energy metabolism	Methane Metabolism					**p=0.099** Serine (+, U), glycine (+, P)

Energy metabolism	Nitrogen Metabolism			**p=0.012** Tyrosine (-), Glycine (+)	**p=0.02** Tyrosine (-, P), Taurine (-, U), Glycine (+, U)
Energy metabolism	Pentose and glucuronate interconversions	**p=0.019** Glucaric Acid (+), Ribitol (+), Arabitol (+), Glyceraldehyde 3-phosphate (-)	**p=0.079** Glutaric Acid (+), Ribitol (+), Arabitol (-)		
Energy metabolism	Pyruvate metabolism	**p=0.023** Acetylphosphate (-), Oxaloacetate (-), Malate (+)	**p=0.022** Homocitrate (+), Acetylphosphate (-), Malate (+)		
Nucleic Acid Metabolism	Purine metabolism		**p=0.096** Adenosine (+), Aconitate (+), Oxalate (-), Cyclic AMP (+)		
Vitamin Metabolism	Biotin Metabolism				**p=0.012** Biotin (+, U), Lysine (-, P)
Vitamin metabolism	Riboflavin metabolism	**p=0.062** Ribitol (+), Hydroquinone (+)			
Vitamin metabolism	Thiamine metabolism			**p=0.005** Tyrosine (-), Glycine(+)	**p=0.054** Tyrosine (-, P), Glycine(+, P)

Table 4. Pathways enriched for U-tAs associated metabolites in plasma (P) and urine (U) of diabetic and non-diabetic individuals. Increases or decreases in metabolite levels are indicated with (+) or (-).

1.5 Discussion

A consensus exists that moderate-to-high chronic exposures to arsenic are associated with an increased risk of diabetes [118]. However, questions remain about pathophysiological processes that underlie this disease and about the phenotype of diabetes developed in the presence of arsenic exposure. One way of answering these questions is the identification of metabolic shifts associated with arsenic exposure among diabetic and non-diabetic individuals. Here we examined plasma and urine metabolites that were altered in response to arsenic exposure in both non-diabetic and diabetic individuals in our Chihuahua cohort where the association between arsenic exposure and prevalent diabetes was recently established [123, 124, 132]. These metabolites and their corresponding pathways were then compared to identify features that were common or unique to the non-diabetic and diabetic individuals in their response to arsenic exposure, to gain insight into arsenic-associated disease.

We identified a total of 132 urinary and plasma metabolites that were associated with U-tAs in either non-diabetic or diabetic individuals, with 103 urinary metabolites and 32 plasma metabolites. Approximately one third of the urinary metabolites that were U-tAs associated (31/103, 30%) were identified as common to both non-diabetic and diabetic subjects. It was interesting to note that in contrast to urine, no overlap was observed in plasma, highlighting the non-concordant results in the matrices measured. The 31 overlapping urinary metabolites include but are not limited to metabolites that broadly play a role in amino acid metabolism (aspartic acid), the TCA cycle (malate, cAMP) and pyruvate metabolism (acetylphosphate). It is important to note that all of these metabolites were changed similarly in their levels between non-diabetic and diabetic subjects demonstrating a broad, disease-independent response to arsenic exposure. As these pathways are critical for energy processing, including adenosine triphosphate (ATP)

production [136], alterations of these pathways could indicate impacts of arsenic exposure on cellular energy production. In addition, the data suggest that effects of arsenic exposure within the shared pathways may differ between diabetic and non-diabetic subjects. For example, the effects on TCA cycle in diabetics included shifts in succinate and fumarate while isocitrate and oxaloacetate were shifted only among non-diabetics; shifts in the concentrations of another two TCA cycle metabolites, aconitic acid and malate, were shared by both conditions. It is known that trivalent iAs, at high doses, can inhibit pyruvate dehydrogenase by binding directly [137]. This mode of action has also been suggested to decrease ATP production.

In the context of disease specificity, we identified a set of 59 U-tAs-associated metabolites that were unique to the diabetic individuals, specifically 42 U-tAs-associated urinary metabolites and 17 U-tAs-associated plasma metabolites. Among these diabetes-specific metabolites were those that play a role in two major areas of human metabolism: (1) the TCA cycle and energy related pathways (succinate, fumarate, taurine), and (2) amino acid metabolism (glycine, serine, methionine, lysine, proline, 5-aminolevulinic acid, glutaric acid, carnitine, tyrosine, phenylglyoxylic acid). Within this group of metabolites are also those associated with metabolism of three major B vitamins: biotin, riboflavin and thiamine. Notably, these vitamins are known for their roles in both the energy and amino acid metabolism. Additionally, both deficiencies for riboflavin and thiamine have been shown to be associated with increased incidence of skin lesions in human populations [138]. Also, methionine and thiamine, in conjunction with other antioxidants, have been shown to reverse the oxidative stress burden in target organs of mice exposed to arsenic [139].

While diabetes developed in the presence of chronic exposure to arsenic has been often referred to as Type-2 diabetes, an exact disease phenotype is only poorly characterized. When

we compared the 59 U-tAs-associated metabolites unique to diabetic individuals in the Chihuahua cohort with the previously published Type 2 diabetes-associated metabolites [115, 140, 141] or metabolites that predict the risk of Type 2 diabetes [141] we found only 12 shared metabolites: 3-hydroxy-3-methylglutaric acid, carnitine, citrulline, delta-hydroxylysine, glycine, lysine, malate, methionine, proline, succinate, tyrosine, and taurine even though all of metabolites were represented [115]. Thus, most of the metabolites that shifted in the urine or plasma of diabetic individuals in response to arsenic exposure were not among the Type 2 diabetes-associated metabolites reported in previous studies. These results point to a unique phenotype of arsenic-associated diabetes apparent at the level of the metabolome. It is possible that the U-tAs-associated metabolites unique to diabetic individuals represent a metabolomic fingerprint of arsenic-associated diabetes.

While an apparent metabolomics phenotype of arsenic-associated diabetes begins to emerge, this study is not without limitations. Most importantly, it was not possible to establish whether diabetic individuals' metabolomics differences were due to diabetes developing as a result of arsenic exposure or if diabetic individuals respond differently to arsenic. The response of an individual to environmental exposures is multifactorial. For this reason, the analysis performed controlled for factors known to influence disease such as age, BMI, and gender. As with similar study designs, it is not possible to assess the roles played by genetic susceptibility, diet and other lifestyle factors. Another limitation of this study was U-tAs was not strongly correlated to DW-iAs suggesting the potential for additional sources of iAs exposure, such as diet and occupational exposures. In addition, it is not possible to directly compare the metabolomes between diabetic and non-diabetic individuals as they were matched on levels of arsenic exposure. Further investigations, including both population-based and laboratory models,

are necessary to determine perturbation of these metabolites is a potential mechanism by which arsenic influences diabetes status.

Taken together there are three major findings from this study: first, we have identified more than 100 metabolites that are altered in urine and plasma in response to arsenic exposure. Second a set of these metabolites was identified that differentiates diabetic individuals from non-diabetic individuals. Third, only a minimal overlap was observed between previously identified Type 2 diabetes-associated metabolites and the diabetes-specific arsenic exposure-associated metabolites, representing a putative fingerprint of arsenic-associated diabetes. These data provide insight into the complex mechanism of action of arsenic-associated disease in human populations with relevance to millions around the globe.

CHAPTER 2: METHYLATED URINARY ARSENICALS TIED TO METABOLOMIC PROFILES IN DIABETIC AND NON-DIABETIC INDIVIDUALS EXPOSED TO ARSENIC IN MEXICO

2.1 Overview

Exposure to inorganic arsenic (iAs) is associated with diabetes mellitus (DM), with alterations apparent at the level of the metabolome. As demonstrated in the previous chapter, arsenic exposure, as measured by total urinary arsenic (U-tAs) is associated with alterations of the metabolome in both diabetic and non-diabetic individuals. More recently it has been shown that arsenic metabolism is associated with risk of disease development. Inter-individual differences in levels and proportions of inorganic arsenic, monomethylated arsenic (MMAs) and dimethylated arsenic (DMAs) in urine have all be shown to impact disease susceptibility in numerous populations. In the case of DM, increased risk of disease development has been associated with increased proportions and levels of DMAs, as measured in urine across multiple cohorts.

The goal of this work was to determine whether metabolomics profiles associated with percentages of arsenicals in urine would be similar or different between diabetic and non-diabetic subjects. The percentages of iAs, MMAs, and DMAs in urine were calculated for 90 diabetic and 86 non-diabetic individuals. Using multivariable linear regression, we then assessed alterations in metabolomics profiles in relationship to each of the arsenical percentages.

Additionally, the profiles identified in relation to the arsenical measures were compared to those identified in association with U-tAs for similarities and differences.

Of 426 unique metabolites tested, 89 urinary and 80 plasma metabolites displayed significant relationships to at least one of the urinary arsenicals. We found associations with metabolites previously associated with diabetes among non-diabetic individuals and unique alterations in metabolomics profiles of diabetic individuals. These results highlight there is significant impact of iAs metabolism on metabolomic profiles of diabetes and non-diabetes individuals as diabetic individuals displayed more shifts in urinary metabolites while non-diabetic individuals displayed more alterations in plasma metabolites. Furthermore, these results demonstrate the important role of arsenic metabolism in disease development as many of the %DMAs associated metabolites have been previously linked to diabetes development.

2.2 Study Objectives

In this study, impacts of arsenic metabolism on metabolomic profiles were assessed. The 426 unique urine and plasma metabolites identified previously were tested against percentages of arsenic metabolites, inorganic arsenic (iAs), monomethylated arsenic (MMAs) and dimethylated arsenic (DMAs) in urine using multivariable linear regression. We determined arsenical-associated metabolomics profiles separately for diabetic and non-diabetic individuals. We then used pathway analysis to examine the context related to the changes we observed in metabolite alterations. The findings of this research suggest that arsenic metabolism maybe associated with diabetes development in the presence of arsenic exposure.

2.3 Materials and Methods

2.3.1 Measurement of Speciated Arsenicals

The concentrations of inorganic and methylated As species in urine were measured using hydride generation-atomic absorption spectrometry coupled with a cryotrap (HG-CT-AAS)[125]. As stated previously Arsenic Species in Frozen Human Urine (SRM 2669; National Institute of Standards and Technology, Gaithersburg, MD) was used as the certified standard reference material. Concentrations of iAs species measured in SRM 2669 by HG-CT-AAS ranged from 86.7 to 106.4% of the certified values. The limit of detection (LOD) for iAs species in urine was 0.01 µg As/L. The pattern of iAs metabolism was characterized using the percentage of tAs present as DMAs, MMAs, and iAs.

2.3.2 Statistical Analyses

For this analysis percentages of each of the arsenicals, %iAs, %MMAs, and %DMAs, were assessed for associations with the abundance levels of 221 plasma metabolites and 294 urinary metabolites using Partek® Genomics Suite™ Software (St. Louis, MO) (Table 2). Multivariable linear regression analyses were used to assess the relationship between the 426 unique metabolites profiled between the urine and plasma matrices and each of the arsenicals. The analyses were conducted separately for diabetes and non-diabetes individuals to assess differences and similarities between groups. We controlled for covariates with known associations with diabetes and arsenic exposure, specifically drinking water iAs, BMI, age and sex. Statistical significance was set at $p<0.05$, $q<0.2$ to maintain comparability to our previously published study.

2.3.3 Metabolic pathway analysis

Canonical metabolic pathway analysis was performed for statistically significantly metabolites using the pathway analysis tool within the MetaboAnalyst package. This tool maps metabolites onto KEGG pathways and uses a Fisher's Exact Test to determine enrichment of molecules and a Relative-between Centrality test to determine the impact of the alterations. Human Metabolome Database (HMDB) identifiers were used for these analyses as they provided greater coverage of metabolites than did the metabolite names. Statistical significance was set at $p<0.05$ for the pathways analysis, as metabolite lists were pre-filtered

2.4 Results

2.4.1 Metabolomic profiles associated with arsenic metabolites in urine and plasma

We assessed the relationships among the indicators of iAs metabolism, namely inorganic arsenic (iAs), monomethylated arsenic (MMAs) and dimethylated arsenic (DMAs) in urine and metabolomics profiles in urine and plasma. We identified a total of 80 urinary metabolites and 89 plasma metabolites that were associated with at least one of the urinary arsenicals (e.g. %iAs, %MMAs, %DMAs) in either diabetes or non-diabetes individuals (Table 5, Supplemental Table 3). Of the 80 urinary metabolites, n=38 were identified in non-diabetes individuals and n=56 were identified in non-diabetes individuals (Table 5). Among the urinary metabolites identified in non-diabetes individuals, n=18 were associated with %iAs, n=16 were associated with %MMAs and n=24 were associated with %DMAs (Table 5). In diabetes individuals, n=28 were associated with %iAs, n=27 were associated with %MMAs, and n=19 were associated with %DMAs (Table 5). Of the 89 plasma metabolites, n=52 were identified in non-diabetes individuals and n=37 were identified in diabetes individuals (Table 5). Among the plasma metabolites identified in non-DMs,

n=24 were associated with %iAs, n=27 were associated with %MMAs and n=38 were associated with %DMAs (Table 5). In diabetes individuals, n=17 plasma metabolites were associated with %iAs, n=16 metabolites were associated with %MMAs and n=18 metabolites were associated with %DMAs (Table 5).

	Non-Diabetic Individuals (n=86)	**Diabetic Individuals (n=90)**
Urine (n=80 metabolites)	**38**	**56**
%iAs	18	28
%MMAs	16	27
%DMAs	24	19
Plasma (n=89 metabolites)	**52**	**37**
%iAs	24	17
%MMAs	27	16
%DMAs	38	18

Table 5. Number of metabolites identified in association with %iAs, %MMAs, and %DMAs in urine and plasma for non-diabetic and diabetic individuals.

2.4.2 Comparison of metabolomic profiles associated with arsenic metabolites in urine and plasma

When comparing the altered metabolomics profiles in diabetes and non-diabetes individuals we observed little overlap (Figure 3). Among urinary metabolites, two %iAs-associated, two %MMAs-associated, and one %DMAs-associated metabolite were shared between diabetes and non-diabetes individuals (Figure 4a). Similarly, among plasma metabolites five %iAs-associated, one %MMAs associated were shared between diabetic and non-diabetic individuals (Figure 4b). No %DMAs-associated metabolites were shared between diabetes and non-diabetes individuals (Figure 4b).

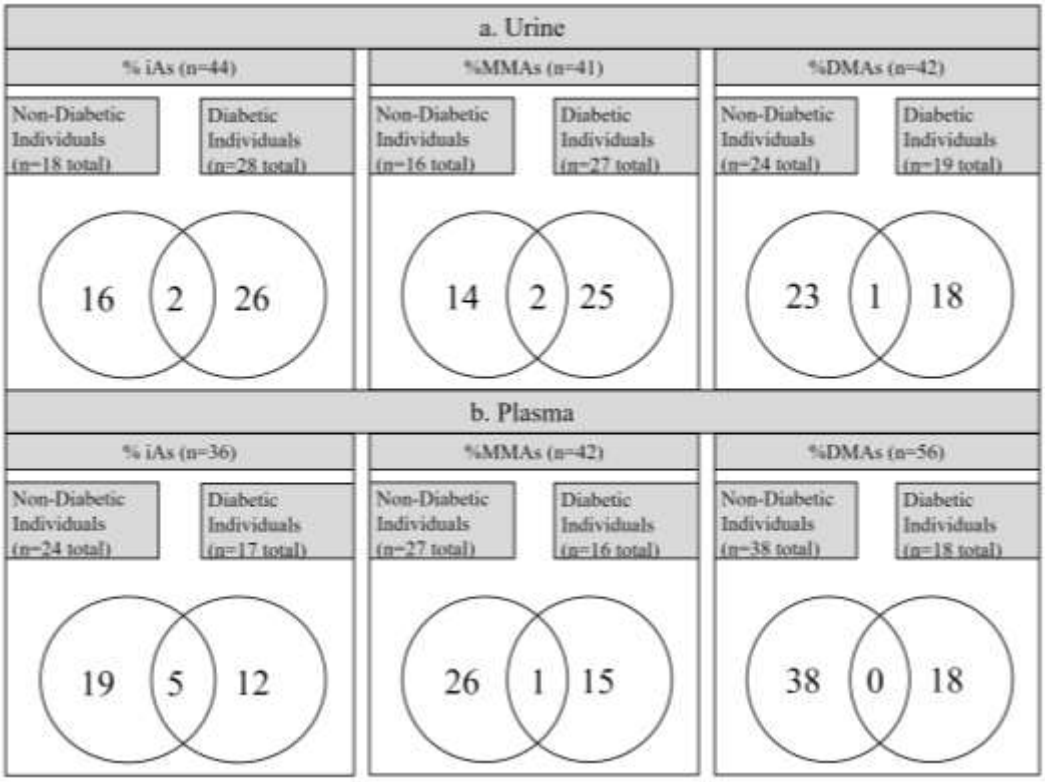


Figure 4. Comparison of urinary (a) and plasma (b) associated with percentages of arsenic metabolites in urine between diabetic and non-diabetic individuals.

We also sought to understand the relationship between the arsenical profiles. We found substantial overlap between the profiles associated with %iAs and %DMAs and those associated with %MMAs and %DMAs in urine and plasma (Figure 5). Interestingly, there were only 4 overlapping metabolites in both urine and plasma between the profiles predicted by %MMAs and %iAs (Figure 4, Supplemental Table 3). Furthermore, there were eight metabolites shared between all three metabolomic profiles in both urine and plasma. In non-diabetic individuals, there were a total 7 urinary and 8 plasma metabolites uniquely associated with %iAs, 7 urinary and 6 plasma metabolites associated with %MMAs and 5 urinary and 6 plasma metabolites associated with %DMAs (Figure 5). Interestingly among the plasma metabolites which overlapped between %MMAs and %DMAs in diabetic individuals, there were a total 17 urinary and 9 plasma metabolites uniquely associated with %iAs, 19 urinary and 9 plasma metabolites

associated with %MMAs and 4 urinary and 3 plasma metabolites associated with %DMAs (Figure 5). Among the profiles, %MMAs has the most uniquely associated metabolites, with a total of 13 %MMAs unique metabolites in non-diabetic and 28 metabolites in diabetic individuals (Figure 5). Conversely, %DMAs has the least uniquely associated metabolites with 11 %DMAs-unique metabolites in non-diabetic individuals and 7 metabolites in diabetic individuals (Figure 5).

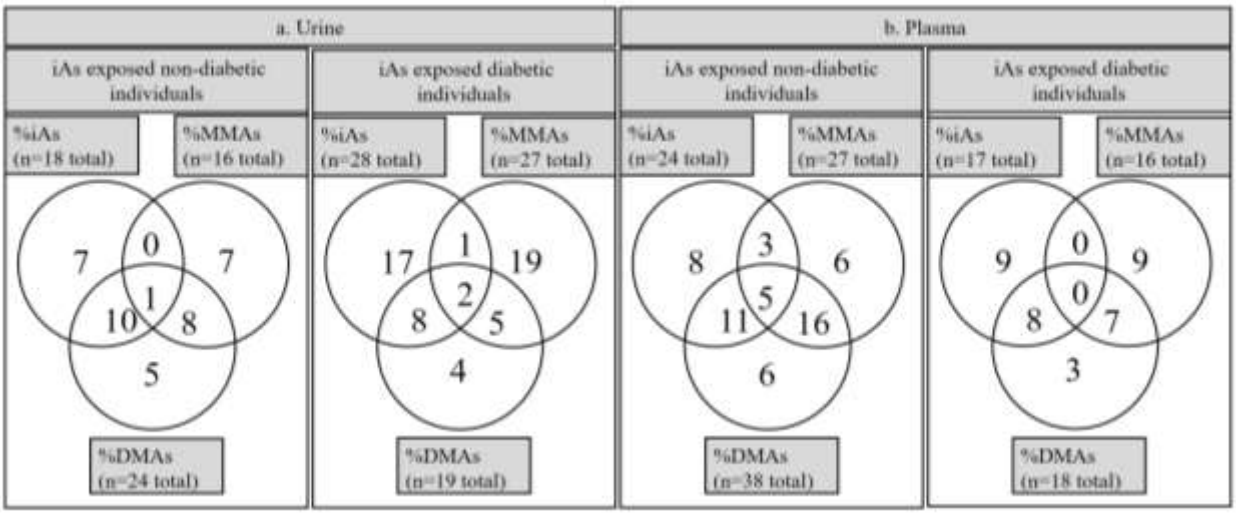


Figure 5. Comparison of urinary (a) and plasma (b) metabolites between arsenical associated percentages in diabetic and non-diabetic individuals.

2.4.3 Pathway analysis of %iAs associated metabolites.

We identified a set of pathways in association with arsenic exposed non-diabetic and diabetic individuals in association with each arsenical for the urine and plasma matrices separately. In the case of %iAs, we identified no pathways enriched by the 18 urinary metabolites and two pathways enriched by the 24 plasma metabolites in non-diabetic individuals (Table 6). In contrast to this, we identified a total of five pathways enriched by the 28 urinary metabolites and one pathway enriched by the 17 plasma metabolites in diabetic individuals. We

saw no overlap of pathways between diabetic and non-diabetic individuals exposed (Table 6). The majority of pathways we identified were either associated with carbohydrate metabolism (Fructose and mannose metabolism, TCA Cycle) and amino acid metabolism (Cyano-amino acid metabolism, alanine, aspartate and glutamate metabolism, and aminoacyl-tRNA biosynthesis).

Pathway Name	# Molecules	p-value	Impact
Non-Diabetic Individuals			
Urinary Metabolites			
-			
Plasma Metabolites			
Pantothenate and CoA biosynthesis	2	0.0074642	0.02002
Fructose and mannose metabolism	2	0.022631	0.0545
Diabetic Individuals			
Urinary metabolites			
Cyanoamino acid metabolism	2	0.006634	0
Citrate cycle (TCA cycle)	2	0.010308	0.06957
Alanine, aspartate and glutamate metabolism	2	0.014695	0.02279
Aminoacyl-tRNA biosynthesis	3	0.019664	0.05634
Methane metabolism	2	0.028502	0.01751
Plasma Metabolites			
Pyrimidine metabolism	3	0.00048	0.07207

Table 6. Pathways enriched for by %iAs associated metabolites in urine and plasma in diabetic and non-diabetic individuals

2.4.4 Pathway analysis of %MMAs associated metabolites

Enrichment of %MMAs associated metabolomic profiles revealed one pathway enriched by the 16 urinary metabolites and three pathways enriched by the 27 plasma metabolites in non-diabetic individuals (Table 7). In diabetic individuals, we identified a total of seven pathways enriched by the 27 urinary metabolites and five pathway enriched by the 16 plasma metabolites (Table 7). We saw overlap of pathways between diabetic and non-diabetic individuals exposed: Phenylalanine, tyrosine and tryptophan biosynthesis and Phenylalanine metabolism were enriched in both groups (Table 7). The majority of pathways we identified were either associated

with amino acid metabolism (Phenylalanine metabolism, phenylalanine, tyrosine and tryptophan metabolism, valine, leucine and isoleucine metabolism, alanine, aspartate and glutamate metabolism, D-glutamine and D-glutamate metabolism and aminoacyl-tRNA biosynthesis).

Pathway Name	# Molecules	p-value	Impact
Non-Diabetic Individuals			
Urinary Metabolites			
Phenylalanine metabolism	2	0.020028	0
Plasma Metabolites			
Phenylalanine, tyrosine and tryptophan biosynthesis	3	0.0012644	0.008
Valine, leucine and isoleucine biosynthesis	3	0.0012644	0.05194
Propanoate metabolism	3	0.0027115	0.00134
Aminoacyl-tRNA biosynthesis	4	0.0028846	0
Nitrogen metabolism	3	0.0037066	0
Valine, leucine and isoleucine degradation	3	0.0039859	0.06442
Phenylalanine metabolism	3	0.0055747	0.11906
Citrate cycle (TCA cycle)	2	0.011396	0.01446
Diabetic Individuals			
Urinary Metabolites			
Phenylalanine metabolism	3	0.004096	0.02373
Phenylalanine, tyrosine and tryptophan biosynthesis	2	0.016601	0.00738
Ubiquinone and other terpenoid-quinone biosynthesis	2	0.028637	0.04886
Plasma Metabolites			
Alanine, aspartate and glutamate metabolism	2	0.004085	0.02279
Butanoate metabolism	2	0.011141	0.0048
Glyoxylate and dicarboxylate metabolism	2	0.017112	0.00686
Synthesis and degradation of ketone bodies	1	0.024695	0
D-Glutamine and D-glutamate metabolism	1	0.044854	0

Table 7. Pathways enriched for by %MMAs associated metabolites in urine and plasma in diabetic and non-diabetic individuals

2.4.5 Pathway analysis of %DMAs associated metabolites

Lastly, we performed pathway enrichment on metabolomic profiles associated with %DMAs in urine and plasma for diabetic and non-diabetic individuals. We found that there was one pathway enriched by the 24 urinary metabolites and three pathways enriched by the 38

plasma metabolites in non-diabetic individuals (Table 8). In contrast to this, we identified a total of seven pathways enriched by the 19 urinary metabolites and two pathway enriched by the 18 plasma metabolites in diabetic individuals (Table 8). We saw no overlap of pathways between diabetic and non-diabetic individuals exposed (Table 8). The majority of pathways we identified were either associated with amino acid metabolism (Alanine, aspartate and glutamate metabolism, valine, leucine and isoleucine biosynthesis, and Phenylalanine, tyrosine and tryptophan biosynthesis).

Pathway Name	# Molecules	p-value	Impact
Non-diabetic Individuals			
Urinary Metabolites			
Alanine, aspartate and glutamate metabolism	2	0.0092452	0.06837
Plasma Metabolites			
Citrate cycle (TCA cycle)	3	0.000169	0.13021
Nicotinate and nicotinamide metabolism	2	0.025856	0.02448
Glyoxylate and dicarboxylate metabolism	2	0.032822	0.02811
Diabetic Individuals			
Urinary Metabolites			
Valine, leucine and isoleucine biosynthesis	3	0.0014641	0.05194
Aminoacyl-tRNA biosynthesis	4	0.00348	0
Valine, leucine and isoleucine degradation	3	0.004597	0.06442
Phenylalanine, tyrosine and tryptophan biosynthesis	2	0.02232	0.008
Tryptophan metabolism	3	0.029564	0.06049
Propanoate metabolism	2	0.036288	0
Nitrogen metabolism	2	0.044259	0
Plasma Metabolites			
Synthesis and degradation of ketone bodies	1	0.027137	0
Pyrimidine metabolism	2	0.029086	0.05715

Table 8. Pathways enriched for by %DMAs associated metabolites in urine and plasma in diabetic and non-diabetic individuals

2.4.6 Comparison of pathways enriched for by arsenical-associated metabolomics profiles

When comparing enriched pathways for %iAs, %MMAs, and %DMAs, we find that there are overlaps as well as uniquely associated profiles. Interestingly, the Citric acid cycle, Aminoacyl-tRNA biosynthesis, Phenylalanine, tyrosine and tryptophan biosynthesis, and Alanine, aspartate and glutamate metabolism were shared among all arsenical-associated metabolomics profiles in both diabetic and non-diabetic individuals. Pyrimidine metabolism was enriched for by both %iAs and %DMAs associated metabolomic profiles in Diabetic individuals only. Additionally, there were many commonalities shared between %MMAs and %DMAs in non-diabetic individuals including Valine, leucine and isoleucine synthesis, Nitrogen metabolism and Propanoate metabolism. Among diabetic individuals, Glycoxylate/dicarboxylate metabolism, and Synthesis of ketone bodies were shared between %MMAs and %DMAs. There were no unique pathways among non-diabetic individuals. However, among diabetic individuals, plasma metabolites associated with %MMAs uniquely enriched for D-glutamine and D-glutamate metabolism and Butanoate Metabolism. Additionally, urinary metabolites associated with %MMAs enriched for Nicotinate and nicotinamide metabolism, and urinary metabolites associated with %iAs enriched for Ubiquinone and terpenoid-Quinone biosynthesis.

2.5 Discussion

Given that arsenic-associated diabetes has been previously associated with arsenic metabolism, specifically increased proportions of %DMAs [37, 50], we set out to determine whether arsenic metabolism impacts metabolomic profiles in diabetic and non-diabetic individuals exposed to arsenic. We identified a total of 80 urinary metabolites and 89 plasma metabolites associated with at least one arsenical in either diabetic or non-diabetic subjects. Additionally, there was little overlap between metabolomic profiles associated with arsenicals for diabetic and non-diabetic individuals exposed to arsenic. Additionally, we found that profiles associated with %iAs, %MMAs and %DMAs shared moderate overlap. Lastly we found that the majority of these metabolites enriched for amino acid metabolism and carbohydrate metabolism. However, a distinct group of pathways was uniquely associated with arsenicals in diabetic individuals. Taken together these data suggest that arsenic-metabolism contributes to development of arsenic associated diabetes.

We found there was minimal overlap between diabetic and non-diabetic individuals exposed to arsenic. There were a total of 9 overlapping probes between the metabolites identified in diabetic and non-diabetic subjects. This represents a total of 8% of probes that were shared between diabetic and non-diabetic individuals. This contrasts our previously published profile of U-tAs associated profiles where we saw a 30% of metabolites common to diabetes and non-diabetes individuals [69]. Furthermore, when comparing the arsenical associated metabolomic profiles to each other there was substantial overlap between %MMAs associated metabolomic profiles and %DMAs associated metabolic profiles as well as between %iAs associated metabolomic profiles and %DMAs associated metabolomic profiles. The differences between profiles associated is interesting as this suggests that there may be effects of arsenic metabolism

on other metabolic processes. These results suggest that arsenic metabolism may play a role in disease development as diabetic and non-diabetic individuals separate out more readily when assessing arsenic metabolite associated metabolomic profiles as compared to total urinary arsenic-associated metabolomic profiles.

Pathway analysis revealed that there were substantial alterations associated with amino acid metabolism. This finding mirrors our previous find where amino acid metabolism was a key category altered in association with total urinary arsenic [69]. Additionally, it is interesting as it has been previously shown that arsenic exposure is associated with alterations in amino acid metabolism and similar associations are also associated with development of diabetes. Furthermore, only a few unique pathways were identified. Interestingly these pathways were all found in diabetic individuals. Specifically, among diabetic individuals, plasma metabolites associated with %MMAs uniquely enriched for D-glutamine and D-glutamate metabolism and butanoate Metabolism. Interestingly glutamine/glutamate ratios and butanoate have been associated with traditional type 2 diabetes [142-144]. It has also been shown that enzyme involved in glutamate metabolism are altered by exposure to arsenic [88, 145]. We also observed that urinary metabolites associated with %MMAs enriched for Nicotinate and nicotinamide metabolism, which has been implicated in glucose control and is of interest in the treatment of diabetes [146-148]. Interestingly, it has also been shown that arsenic perturbs enzymes involved nicotinamide and nicotinate metabolism [121, 149] and supplementation of nicotinamide reduces arsenic-induced skin cancer [150]. Interestingly these pathways are linked to arsenic as well as development of diabetes. This suggests that arsenic metabolism may have effects of processes related to arsenic-associated metabolism.

As %DMAs have been associated with diabetes [37, 50], we were particularly interested in examining the associated metabolites associated with these pathways. Pathway analysis of the 37 %DMAs-associated metabolites revealed an enrichment for the citric acid (TCA) cycle. In prior work, we previously observed disruption of metabolites within the TCA cycle in association with total urinary arsenic (U-tAs) in diabetes individuals [69]. In the present study, decreases in the levels of TCA intermediates fumarate, isocitrate, and cis-Aconitic acid were observed in relation to %DMAs. In our previous work, U-tAs was associated with increases of fumarate and succinate in diabetes individuals [69]. These results suggest a general dysregulation of the TCA cycle in relation to iAs exposure, supported by prior studies as well [151]. Our results are further supported by research that demonstrates diabetes subjects have decreased muscular TCA cycle flux, which have been attributed to mitochondrial dysfunction, and enzymatic disruptions of key TCA cycle enzymes [142, 152]. Taken together, these data suggest that differences in iAs-metabolism may be related to dysregulation of the TCA cycle in exposed diabetes individuals.

In addition to the examination of iAs-associated metabolomic profiles in diabetes subjects we also examined profiles in non-diabetes individuals. Interestingly, increases in %DMAs were associated with increases in levels of the following five amino acids: valine, leucine, isoleucine, phenylalanine, and tyrosine. These metabolites are of special interest as their increased serum levels have been associated with increased odds of diabetes development prior to diagnosis [153, 154]. Interestingly, diabetes individuals did not display differences in these amino acids levels in relation to iAs metabolism indicators. As mechanistic studies have linked amino acid metabolism to insulin resistance and subsequent development of DM, these data could provide insight into which mechanisms are relevant to iAs-induced diabetes [122]. Future research could focus on whether these amino acids serve as biomarkers for diabetes in iAs-exposed individuals.

Our results demonstrate that when analyzed in the context of methylated urinary arsenicals proportions, there are substantial differences between metabolomics profiles of diabetes and non-diabetes individuals. These result further substantiate that inter-individual differences in iAs metabolism is important to understand iAs-associated disease. Furthermore, we demonstrate that metabolomic changes observed in association with %DMAs reflect critical biochemical pathways. These pathways provide novel information on the mechanisms by which %DMAs may be tied to DM. Metabolomics is a powerful tool for identifying mechanisms underlying iAs-associated disease, and identifying potential disease biomarkers.

CHAPTER 3: ARSENIC-3-METHYLTRANSFERASE (AS3MT) GENOTYPE IS ASSOCIATED WITH UNIQUE METABOLOMIC PROFILES IN DIABETIC AND NON-DIABETIC INDIVIDUALS EXPOSED TO ARSENIC IN MEXICO

3.1 Overview

The enzyme Arsenic-3-Methyltransferase (*AS3MT*) is primarily responsible for metabolism of inorganic arsenic. Arsenic metabolism is an important indicator of disease susceptibility with %DMAs being associated with diabetes susceptibility. Interestingly genotype of *AS3MT* has been associated with alterations in percentages of arsenic metabolites. Furthermore, a subset of these are associated with increased risk for numerous iAs-associated diseases including Diabetes Mellitus (DM). Taken together these data support the hypothesis that the metabolism of iAs into its methylated metabolites influences diabetes risk.

Given that iAs-associated diabetes is also associated with differences at the level of the metabolome, as well as observed differences between metabolomics profiles associated with arsenic metabolism, we set out to understand the relationship between genotype of *AS3MT* and the metabolome. To elucidate potential pathways that *AS3MT* might influence resulting in inter-individual differences in diabetes risk, metabolomic profiles from diabetes and non-diabetes individuals were analyzed in both a stratified and non-stratified manner, for associations with six single nucleotide polymorphisms (SNPs) of *AS3MT* were analyzed in association with the 426 urinary and plasma metabolites assessed during untargeted metabolomic assessment.

A total of four SNPs, rs17881215, rs3740393, rs3740390 and rs10748835 were associated with 36 urinary and plasma metabolites in stratified and non-stratified modeling. Many of these metabolites have been previously associated with type 2 DM. Additionally, these metabolites were associated with diabetes associated processes such as glucose metabolism, amino acid metabolism and riboflavin metabolism. Given the relationship of these metabolites and *AS3MT*, it is possible that arsenic metabolism impacts metabolic processes related to diabetes thus influencing inter-individual susceptibility to arsenic-induced DM. These metabolites could help inform a mechanistic understanding by which arsenic is able to induce diabetes in humans, as well as serve as potential pathways for targeting of intervention strategies.

3.2 Study Objectives

In the present study, metabolic impacts of *AS3MT* not related directly to arsenic metabolism were assessed. This was done by integrate metabolomic profiles and genotype data sets. There were a total of 123 individuals, 67 non-diabetic and 56 diabetic individuals, for analysis. Individuals were coded has having zero, one or two copies of the variant allele for each SNP. Their SNP profile was then compared individually to each of the 426 urinary and plasma metabolites. This was done in both a non-stratified and stratified models. Taken together the results of this analysis suggest that arsenic metabolism may impact metabolic processes related to diabetes development and thus provide a mechanistic link between arsenic-metabolism and disease susceptibility.

3.3 Materials and Methods

3.3.1 Cohort Selection

A total of 1,165 adults (≥18 years old) with a minimum of 5-year uninterrupted residency in the study area were recruited between 2008 and 2012 [123, 124]. Individuals were excluded if pregnant, occupational exposed to arsenic, or reported kidney or urinary tract infection as these conditions may affect profiles of arsenic metabolites in urine. Drinking water samples were obtained from each subjects' households, and an interviewer-administered study questionnaire was used to record data on residency, occupation, drinking water sources and use, smoking, alcohol consumption, and medical history. During medical exams, spot urine and fasting venous blood were collected and an oral glucose tolerance test with blood drawn 2 hours after a 75 g glucose dose. Plasma from both fasting and two-hour blood samples and urines were immediately frozen and stored at -80^{o}C. Measures of body weight and height were obtained during the exams and used to calculate body mass index (BMI). All procedures involving human subjects were approved by the IRBs of UNC Chapel Hill and Cinvestav-IPN, and all participants signed a written consent to participate in the study.

As described previously, 176 individuals, comprising 90 diabetic and 86 non-diabetic individuals, had been selected and profiled by metabolomics assessment [69]. A separate subset of individuals (n=543 individuals) had been previously assessed for AS3MT genotypes [155]. Between the two groups, 123 individuals, 67 non-diabetic and 56 diabetic individuals, had both genotyping and metabolomics profiles available for assessment.

3.3.2 Genotyping assessment

DNA was isolated from venous blood collected during the oral glucose tolerance test using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. A total of eight *AS3MT* single nucleotide polymorphisms (SNPs) were selected for analysis given their relationships to arsenic metabolism and or susceptibility to toxicity. The SNPs selected for analysis were: rs35232887, rs34556438, rs11191439, rs17881215, rs3740393, rs3740390, rs11191453, and rs10748835. Predesigned TaqMan (Applied Biosystems, Carlsbad, CA, USA) assays were used to assess alleles of the SNPs at either the Mammalian Genotyping Core (UNC, Chapel Hill, North Carolina, USA) or in the Mendez laboratory (rs17181215). The ABI Dual384-Well GeneAmp PCR System 9700 and ABI PRISM 7900 HT-Sequence Detection System from Applied Biosystems was used for genotyping and the ABI SDS software for data analysis. VNTR variants and rs17881215 were identified by sequencing a PCR-amplified promoter region. Due to genotyping failure and lack of variability, rs35232887 and rs34556438 were not considered for further analysis.

3.3.3 Statistical Analysis

All statistical analyses were carried out using Partek Genomic Suite (St. Louis, MO). SNP genotypes were numerically coded as zero (wild type homozygote, zero copies of the minor/less common allele), one (heterozygote, one copy of the minor allele) or two (variant homozygote, two copies of the minor allele).

Multivariable linear regression was performed to examine the relationships between each of the six AS3MT SNPs, to each of the 294 urinary and 221 plasma metabolites. This allowed for comparisons to the primary predictor of AS3MT genotype, while controlling for the effects of

several important cofounders known to affect arsenic metabolism: age, sex, BMI, and drinking water iAs levels. The data were analyzed for outliers and determined not to be influenced by outliers. Five models were constructed and tested. The primary model (Model I) included all individuals testing AS3MT genotype as a predictor. Additionally, given that we observed different metabolomics profiles for diabetic and non-diabetic subjects in association with arsenic exposure, subjects were stratified as non-diabetic individuals (Model II) and diabetic individuals (Model III). Beta (β) coefficients were calculated representing the estimated change in metabolite levels given the addition of one copy of the minor allele. To determine these, the referent group was the wild type homozygous group carrying zero copies of the minor allele, was compared to the heterozygote group carrying one copy of the minor allele. Statistical significance was set at $p \leq 0.05$, $q<0.1$.

3.4 Results

3.4.1 Demographic Assessment

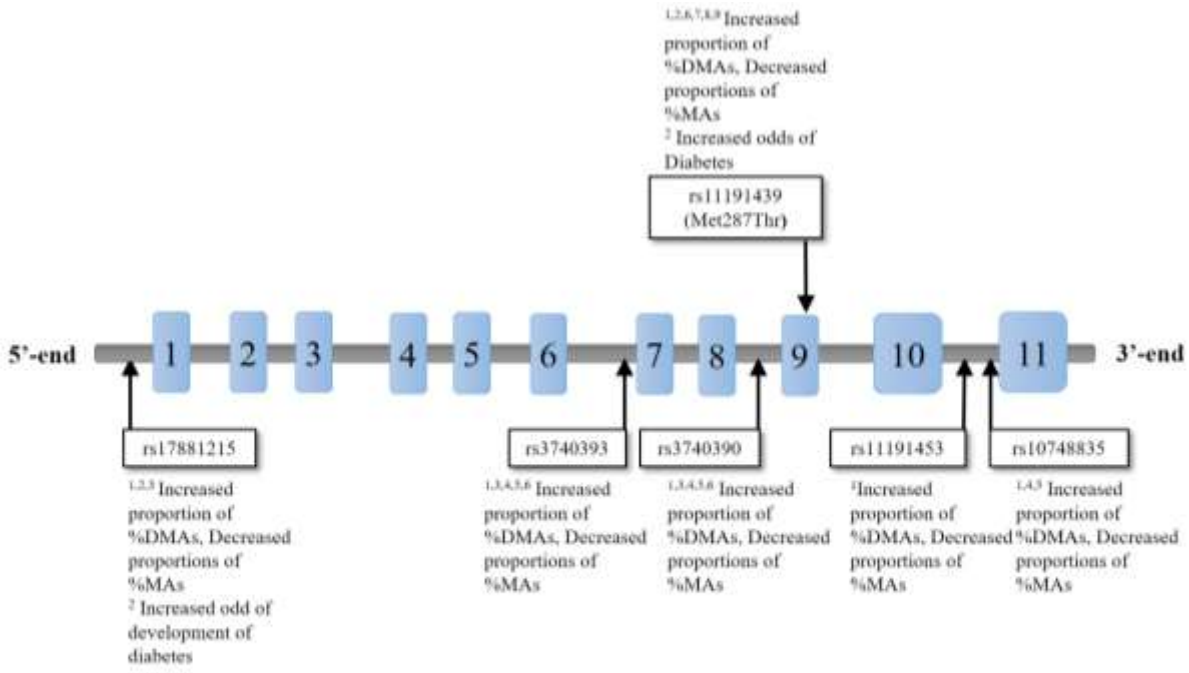
There were 123 individuals (67 non-diabetic and 56 diabetic individuals) profiled for this analyses. These individuals were similar in terms of age (non-diabetic individuals mean=50.0, diabetic individuals mean=51.5) sex (female non-diabetic individuals-71.6%, female diabetic individuals-76.8%), and BMI (non-diabetic individuals mean=29.2, diabetic individuals mean=29.8) (Table 9). The individuals did differ substantially in their fasting plasma glucose (FPG) (non-diabetic individuals mean= 88.7, diabetic individual mean=167.1) and 2-hour blood glucose level (2HPG) (non-diabetic individuals mean=110.7, diabetic individuals mean=213.7). Their levels of arsenic exposure were similar with non-diabetics exposed to 67.3 µg/L iAs in drinking water and diabetic individuals exposed to 78.2 µg/L drinking water on average (Table 9).

	All Individuals (n=123)	**Non-Diabetic Individuals (n=67)**	**Diabetic Individuals (n= 56)**	**p-value**
Sex				
Male	32 (26.0%)	19 (28.4%)	13 (23.2%)	
Female	91 (74.0%)	48 (71.6%)	43 (76.8%)	
Age	50.7 (18-79)	50.0 (18-78)	51.5 (23-79)	0.555
BMI	29.5 (18.3-43.6)	29.2 (19.2-43.6)	29.8 (18.3-43.1)	0.557
FPG (mg/dL)	124.4 (49.5-279)	88.7(49.5-121)	167.1 (69-397)	<0.001
2HPG (mg/dL)	157.2 (50-437)	110.7 (50-176.5)	213.7 (50.5-437)	<0.001
DW-iAs (µg/L)	72.3 (<LOD-292.9)	67.3 (<LOD-284.7)	78.2 (<LOD-292.9)	0.396

Table 9. Demographics for individuals with both in the AS3MT genotyping and metabolomic analysis.

3.4.2 General Polymorphic Characteristics

The individuals analyzed were categorized into one of three categories depending upon their genotypes: wild-type homozygotes (i.e., carriers of two copies of the major allele), variant homozygotes (carriers of two copies of the minor allele) or heterozygotes (carriers of one copy of the major allele and one copy of the minor allele). Five of the tested SNPs are located within intronic regions (rs17881215, 3740393, rs3740390, rs11191453, rs10748835) of *AS3MT* (Figure 6). One non-synonymous SNP present in exon nine alters the amino acid sequence (Met287Thr) of the AS3MT protein (rs1191439). All tested SNPs have been previously associated with increased proportion of DMAs in urine (Figure 6), a risk factor for DM.



1. Xu et al. 2016 2. Drobna et al 2013. 3. Drobna et al 2016 , 4 Engstrom K.S (2011) 5. Meza, M.M et al (2005) 6. Engstrom, K.S. et al (2007) 7. Agusa T et al (2009), 8. Hernadez A, et al (2008) 9. Lindberg A.L, et al (2007)

Figure 6. Location of SNP Positions within the AS3MT gene. Adapted from Drobna 2016

Allelic frequencies were generally consistent with those found in the larger group of individuals analyzed (Xu 2016) (Table 10). All examined SNPs passed the Hardy-Weinberg equilibrium test at $p < 0.05$ (Table 10).

	All Individuals	Non-Diabetic Individuals	Diabetic Individuals	Xu 2016
rs11191439	T: 0.93 (229)	T: 0.95 (127)	T: 0.91(102)	T: 0.91
	C: (0.07) 17	C: 0.05 (7)	C: 0.09 (10)	C: 0.09
rs11191453	T: 0.78 (192)	T: 0.78(104)	T: 0.79 (88)	T: 0.79
	C: 0.22 (54)	C: 0.22 (30)	C: 0.21 (24)	C: 0.21
rs3740393	G: 0.72(178)	G: 0.73 (98)	G: 0.71 (80)	G: 0.75
	C: 0.28 (68)	C: 0.27 (36)	C: 0.29 (32)	C: 0.25
rs3740390	C: 0.74 (183)	C: 0.75 (101)	C: 0.73 (82)	C: 0.77
	T: 0.26 (63)	T: 0.25 (33)	T: 0.27 (30)	T: 0.23
rs10748835	A: 0.57(139)	A: 0.57 (77)	A: 0.55 (62)	A: 0.55
	G: 0.43 (107)	G: 0.43 (57)	G: 0.45 (50)	G: 0.45
rs17881215	G: 0.92 (226)	G: 0.93 (125)	G: 0.90 (101)	G: 0.90
	C: 0.08 (20)	C: 0.07 (9)	C: 0.10 (11)	C: 0.10

Table 10. Allelic Frequency Table and comparison to frequencies found within the larger cohort published in Xu et al 2016 [155]

3.4.3 Model 1: Non-stratified modeling reveals associations between four SNPs and altered metabolism

For each of the six SNPs analyzed, multiple variable linear regression was used to identify associations between *AS3MT* alleles and urine and plasma metabolite levels in both diabetic and non-diabetic subjects. Of the 294 urinary metabolites analyzed and 221 plasma metabolites analyzed, 12 urinary metabolites and one plasma metabolite displayed associations with at least one SNP (Table 11). These 12 metabolites were associated with at least one of the following four SNPs: rs17881215, rs3740393, rs3740390 and rs10748835 (Table 11). Of the urinary metabolites, rs3740390 displayed associations with ten metabolites, including the amino acids arginine and phenylalanine, and the glycolysis intermediate glyceraldehyde-3-phosphate (Table 11). The majority (n=9, 75%) of the metabolites displayed increased levels in carriers of

the variant allele, while only three displayed decreased levels in association with the variant allele. The plasma metabolite identified was dodecanoic acid, and it displayed a positive association with the presence of the variant allele rs10748835 (Table 11).

	Urine Metabolites (β-value)	Plasma Metabolites (β-value)
rs17881215	7-Methyl-indole (0.17)** 5-Hydroxydopamine (0.18)**	-
rs11191439	-	-
rs11191453	-	-
rs3740393	Glyceraldehyde 3-phosphate (0.17)**	-
rs3740390	Myo-Inositol (0.20)** Guanidineacetic acid (0.13)** 2-Hydroxy-3-methylpentanoic acid (0.18)** 3-Methylindole (0.17)** Homovanillic acid (-0.08)** N-methyl-L-histidine (-0.25)** Phenylalanine (0.22)** 5-Aminolevulinic acid (-0.31)** Arginine (-0.31)**	-
rs10748835	-	Dodecanoic acid (0.34)**

Table 11. Statistically significantly associated metabolite-SNP associations in all individuals (n=123). Displayed are β coefficients of the linear regression model indicating the direction and magnitude of the alteration. * $p<0.05$, ** $p<0.05$, $q<0.1$

3.4.4 Model 2: Non-diabetic individuals display unique metabolic alterations associated with the four SNPs previously identified in Model 1

Given that different metabolite profiles have been previously observed for diabetic and non-diabetic individuals, we performed stratified analyses for non-diabetic individuals alone (Table 12). We found two urinary and four plasma metabolites associated with at least one SNP in non-diabetic individuals alone (Table 12). Again the same four SNPs, as detailed above, namely rs17881215, rs11191439, rs370393 and rs3740390 showed association with the metabolites. As observed among the metabolites identified in Model I, glyceraldehyde 3-phospate showed a positive association with rs3740393 and, guanidineacetic acid showed a

positive association with rs374390 (Table 12). In contrast to Model I, among the plasma metabolites, dodecanoic acid showed an association with rs17881215, rather than rs10748835 (Table 11, Table 12). Additionally, three new metabolites were identified as displaying positive associations: tetradecanoic acid was associated with rs17881215, 11-eicosenoic acid was associated with rs17881215 and rs11191439, and rs3740390 was associated with 2,5-dihydroxybenzoic acid (Table 12).

	Urine Metabolites (β-value)	Plasma Metabolites (β-value)
rs17881215	-	Tetradecanoic acid (0.29)** 11-Eicosenoic acid (0.45)** Dodecanoic acid (0.50)**
rs11191439	-	11-Eicosenoic acid (0.44)**
rs11191453	-	-
rs3740393	Glyceraldehyde 3-phosphate (0.21)**	-
rs3740390	Guanidineacetic acid (0.22)**	2,5-dihydroxybenzoic acid (0.36)**
rs10748835	-	-

Table 12. Statistically significantly associated metabolite-SNP associations in non-diabetic individuals (n=67). Displayed are β coefficients of the linear regression model indicating the direction and magnitude of the alteration. * $p<0.05$, ** $p<0.05$, $q<0.1$

3.4.5 Model 3: Diabetic individuals display unique alterations associated with the four SNPs previously identified in Model 1 and Model 2

During stratified analyses, diabetic individuals, in addition to non-diabetic individuals, were tested alone for associations between genotype and metabolomic profiles. Among diabetic individuals we found alterations of 13 urinary metabolites and one plasma metabolite, in associations with two SNPs (Table 13). All 13 urinary metabolites were positively associated with rs17881215, including 7-methyl-indole and 5-hydroxydopamine, which were found in Model 1 (Table 11, 13). Additional metabolites included, oxalic acid, quiolinic acid, ribitol and

riboflavin (Table 13). The plasma metabolite identified, Palmitin, was negatively associated with the presence of the variant allele for rs1078835 (Table 13).

	Urine Metabolites (β-value)	Plasma Metabolites (β-value)
rs17881215	Quinolinic acid (0.46)** 7-Methyl-indole (0.28)** 3-Desoxypentitol (0.27)** Ribitol (0.33)** 3-Methoxytyrosine (0.33)** 4-Hydroxy-proline (0.33)** Phenylacetylglycine (0.46)** Oxalic acid (0.28)** 2,3-Diaminopropionic acid (0.33)** Choline (0.33)** 5-Hydroxydopamine (0.28)** 3-Methylglutaric acid (0.29)** Riboflavin (0.37)**	
rs11191439	-	-
rs11191453	-	-
rs3740393	-	-
rs3740390	-	-
rs10748835	-	Palmitin (-0.08)**

Table 13. Statistically significantly associated metabolite-SNP associations in diabetic individuals (n=56). Displayed are β coefficients of the linear regression model indicating the direction and magnitude of the alteration. * $p<0.05$, ** $p<0.05$, $q<0.1$

3.5 Discussion

It has been previously demonstrated that arsenic-associated diabetes is likely tied to the metabolism of arsenic [37, 50]. The enzyme *AS3MT* is required for, and has been previously associated with alterations in arsenic metabolism and with increased odds of diabetes development [111]. We integrated untargeted metabolomics data and genotypic profiles of diabetic and non-diabetic subjects to understand alterations in arsenic metabolism associated with other metabolic alterations. Specifically, we profiled six *AS3MT* SNPs that had been previously associated with diabetes and increased proportions of DMAs. A total of four SNPs (rs17881215, rs3740390, rs3740393 and rs10748835) were identified to be associated with alterations in 36 urinary and plasma metabolites**.** Of the SNPs associated with metabolites, presence of the variant allele of rs17881215 has been previously associated with diabetes in arsenic exposed individuals [111]. These metabolites are linked to processes we identified as different between diabetic and non-diabetic individuals exposed to arsenic.

Of the metabolites identified several were related to glucose metabolism. Interestingly, SNP rs17881215, were associated with differences in levels of oxalic acid, a component of the citric acid (TCA) cycle that has been previously associated with diabetes. We also identified oxalic acid as altered in relationship to arsenic exposure in diabetic individuals [69]. Additionally, SNP rs3740393 was associated with levels of glyceraldehyde-3-posphate in the non-stratified model and in non-diabetic individuals. This metabolite is an intermediate metabolite of glycolysis, and increased levels are associated with diabetic complications [156]. We had previously identified the TCA cycle as a target of potential interest as it was associated with arsenic exposure in both diabetic and non-diabetic subjects [69].

Several of the identified metabolites identified here in relation to *AS3MT* genotype were related to amino acid metabolites. This is supported by our prior work that showed alterations of amino acids, specifically arginine and proline metabolism, previously identified as arsenic-associated in diabetic and non-diabetic individuals. In the non-stratified model and in non-diabetic individuals alone, we identified levels of the metabolite guanidineacetic acid, a precursor of several amino acids, were associated with SNP rs3740390. Interestingly, we found that presence of the variant allele was associated with increased guanidineacetic acid, and a decrease of its downstream metabolite, Arginine. In support of this finding, high levels of urinary excretion of guanidinoacetic acid have been observed in diabetic rats [157], while decreased arginine has been associated with insulin resistance [158]. Additionally, increased levels of the amino acid phenylalanine were associated with the variant allele for rs3740390. Increased levels of this amino acid have been linked to future development of diabetes [153, 154].

We also identified alterations in vitamin metabolism associated with *AS3MT* genotype. Riboflavin, which we identified as altered in diabetic individuals in relation to arsenic exposure, was associated with rs17881215 [69]. Interestingly, it has been shown that individuals with diabetes have abnormal riboflavin metabolism [159, 160], and we previously showed it to be associated with arsenic exposure in diabetic individuals [69]. Furthermore, it is thought that riboflavin can help improve glucose control [161, 162].

The last group of metabolites are associated with metabolic alterations observed in diabetes. Of interest was, 5-hydroxydopamine, associated with rs17881215, which was associated in the non-stratified model and in diabetic individuals. Treatment of diabetic mice with other hydroxydopamine compounds has been shown to result in decreased food intake, weight loss, decreased glucose levels and improved pancreatic islet granulation [163, 164]. In contrast to this,

rs10748835 was associated with palmitin which has been shown to induce apoptosis in pancreatic beta cells [165], one of the mechanisms associated with arsenic-associate diabetes. A slight decreased was observed in association in carriers of the variant allele, in contrast to our *a priori* hypothesis that these individuals would have higher levels of palmitin. This could be an issue of power, as we had a relatively small sample size. Additionally, increased levels of the plasma metabolites were associated with 11-eicosenoate and tetradecanoic acid were associated with the variant alleles of rs17881215. Both rs17881215 and rs10748835 were associated with dodecanoic acid. Interestingly, increases in all three metabolites have all been previously associated with diabetes [166, 167]. Taken together the various metabolites associated with rs17881215 are associated with various processes known to be altered in diabetes processes, suggesting that there is an association between rs17881215 and alteration of other metabolic processes. Finally, the plasma metabolite associated with rs3740390 variant allele, 2,5-dihydroxybenzoic acid, has been found to be increased in tissues of diabetic rats [168]. The metabolites associated with the presence of the variant alleles of rs3740390 were also associated with increased risk of diabetes development.

In conclusion, the variant allele for all SNPs was associated with differences in the levels of metabolites and metabolic pathways related to diabetes development. These data suggest that arsenic metabolism may impact other metabolic pathways that play a role in diabetes development. These findings can inform mechanistic understandings about arsenic-associated diabetes and the underlying differences that result in inter-individual differences. This study represents a unique integration of genotype and metabolomic profiles to provide insight into potential pathways that are related to arsenic metabolism, and inter-individual susceptibility to diabetes development in the presence of arsenic exposure.

CHAPTER 4: DISSERTATION DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

Metabolomics profiles may provide mechanistic insight into arsenic-associated diabetes

Collectively, the studies presented here are part of my dissertation demonstrate that there is a relationship between arsenic exposure, arsenic metabolism and inter-individual variability that are associated with risk of diabetes development. Specifically, in Chapter 1 we show that arsenic exposure, as assessed by U-tAs, is associated with a unique fingerprint of metabolites among diabetic individuals that are not mirrored by traditional type 2 DM. In Chapter 2, we find that arsenic metabolism, as assessed by %iAs, %MMAs, and %DMAs, are associated with unique metabolomic profiles and separate diabetic and non-diabetic individuals. Finally, in Chapter 3 we demonstrate that genotype for *AS3MT*, the major protein responsible for arsenic metabolism is associated with a set of metabolites many of which are related to type 2 diabetes. These data provide mechanistic understanding, as well as inter-individual differences, for processes involved in diabetes development in the presence of arsenic exposure.

A primary goal of the work in Chapter 1 was to identify arsenic exposure-associated metabolites in diabetic and non-diabetic individuals. This research is important as it is the first study to identify a profile of arsenic associated metabolites in a human population using a metabolomic screening method. Alterations of metabolites in response to arsenic exposure enrich for alterations in metabolic pathways related to energy metabolism, vitamin B metabolism and

amino acid metabolism. The identified pathways suggest potential roles through which arsenic exposure may lead to the development of diabetes in the context of arsenic exposure. It was also noted that a subset of metabolites was uniquely associated with U-tAs in diabetic individuals alone. Additionally, as many of the identified metabolites did not overlap with metabolites previously identified as associated with type 2 diabetes metabolomic profiles, these represent novel targets for study. Taken together the first study is pioneering in its use of metabolomics technology in arsenic exposure studies of human populations, identification of pathway level alterations of the metabolome in the context of arsenic exposure and the identification a U-tAs associated diabetes unique fingerprint.

A major goal of the work in Chapter 2 was to investigates the role of arsenic metabolism on the metabolome. This study shows that arsenical-associated metabolites are more different than U-tAs associated metabolites between diabetic and non-diabetic subjects. Furthermore, we demonstrate that arsenicals are associated with many of the same pathways identified in association with U-tAs-associated metabolites. Specifically, amino acid metabolism and the TCA cycle. Finally, we show that increased levels of %DMAs in non-diabetic individuals are associated with increased levels of five amino acids (leucine, isoleucine, valine, phenylalanine and tyrosine). Increased levels of these amino acids has been shown to be predictive of diabetes status in three separate cohort studies. Taken together, data from the second study demonstrate that arsenic metabolism plays a key role in development of arsenic-associated diabetes. Furthermore, it provides increased mechanistic understanding as to why increased proportions of %DMAs are more strongly associated with likelihood of developing diabetes than U-tAs.

The research presented in Chapter 3 examines inter-individual differences as a susceptibility factor for diabetes development. Given that *AS3MT* is the key enzyme responsible for metabolism of arsenic, and the second study demonstrated the importance of arsenic metabolism on the metabolism, it is probable that genotypic differences in *AS3MT* is associated with risk of diabetes development. We set out to understand the mechanisms by which genotype may confer susceptibility on certain individuals. A set of 36 urinary and plasma metabolites were identified to be associated with allelic composition of four SNPs of *AS3MT*. Many of the metabolites were associated with diabetes development or mechanisms of diabetes development. Additionally, nay of the metabolites were metabolites associated with the pathways associated with both arsenic exposure and arsenic metabolism: energy metabolism, amino acid metabolism and vitamin B metabolism. Taken together, these data suggest that arsenic metabolism, specifically that mediated by *AS3MT,* is important to understanding inter-individual risk associated with diabetes development.

In conclusion, these three studies demonstrate the role of arsenic exposure, metabolism and susceptibility factors related to arsenic metabolism, in altering the metabolome. These data represent some of the first to utilize metabolomic screening in the context of arsenic exposure. The conclusions from these studies inform mechanistic understandings of arsenic-associated diabetes in human populations and potential targets for intervention in the context of arsenic associated DM.

The urinary metabolic fingerprint is associated with arsenic metabolism and *AS3MT* genotype

In Chapter 1, an arsenic-associated diabetes unique fingerprint was identified. We identified a total of 59 metabolites identified in association with arsenic exposure, as assessed by

U-tAs, that were not found in non-diabetic individuals. Additionally, 47 of these metabolites were not found to be associated in metabolomic studies of traditional type 2 diabetes.

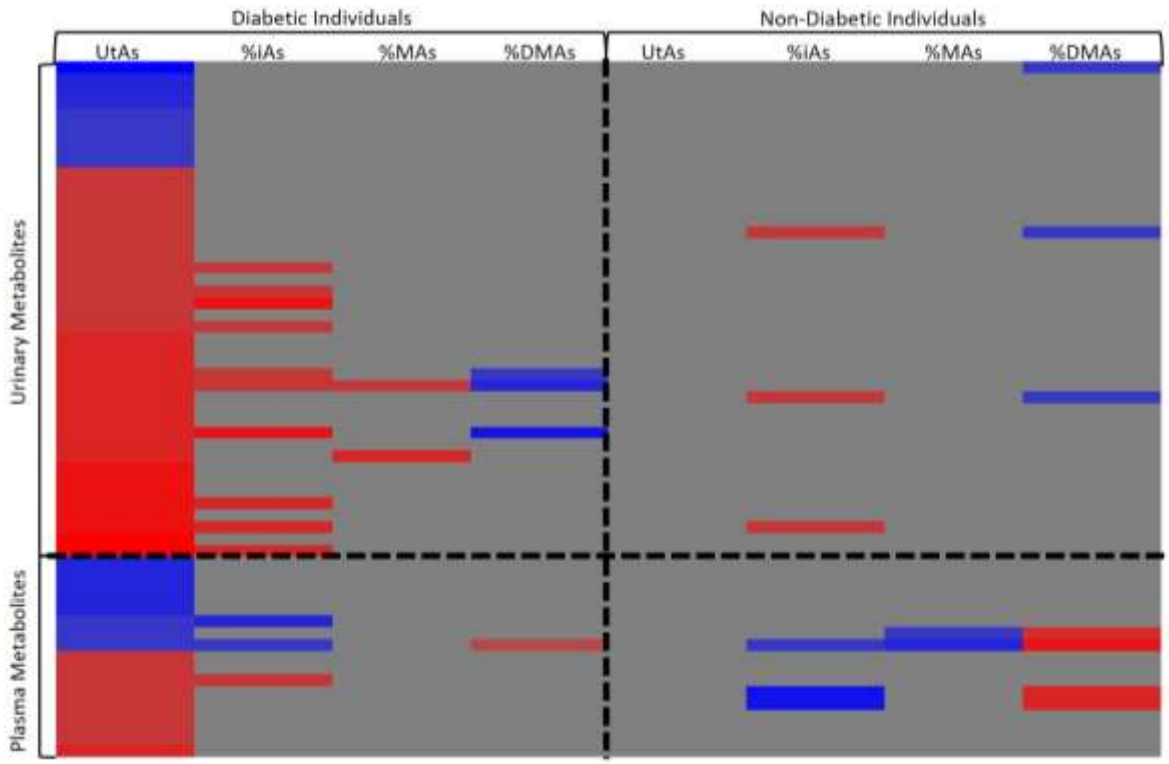


Figure 7. Comparison of U-tAs (exposure) based fingerprint of arsenic-associated diabetes to metabolites found to be significantly associated with each of the arsenical measures in diabetic and non-diabetic subjects.

When we compare these metabolites to those identified in association with arsenical-measures in diabetic and non-diabetic individuals identified in Chapter 2, we find that there are a number of overlaps (Figure 7). Surprisingly, the most overlaps (n=13) were observed in association with %iAs (Figure 7). This was contrary to our *a priori* hypothesis that the most overlaps would be identified in association with %DMAs, as increased levels of %DMAs are associated with risk of diabetes development. Among the metabolites identified in association with %iAs in cases were amino acid related metabolites (3.4-Dehydro-DL-proline, serine), and TCA related metabolites (succinate). Only two metabolites, hexanedioic acid and theritol were

associated %MMAs in case and the fingerprint. Lastly for %DMAs in diabetic individuals, there were four fingerprint metabolites: 3-hydroxy-3methylglutaric acid, hexanedioic acid, 3,3-dimethylglutaric acid and 3,4-dihydro-DL-proline. All of the DMA metabolites were associated in a direction opposite to the association of U-tAs. These metabolites may provide increased information about relationships between arsenic exposure, alterations in arsenic metabolism and diabetes risk.

Interestingly, we found some of the metabolites in our fingerprint were associated with arsenicals in non-diabetic individuals. Interestingly among these there was a strong overlap between those associated with %iAs and %DMAs (Figure 7). There were five fingerprint metabolites shared between the %iAs and %DMAs: Isohomovanillic acid, 5-methylcytosine, gluconic acid, 3,4-Dehydro-DL-proline and ribofuranose. 3,4-Dehdyro-DL-proline was also associated with %MMAs. Tyrosine was associated with %DMAs and %MMAs. There was one metabolite, 5-methylcrotonylglycine, uniquely associated with %iAs. Similarly, there was one metabolite, deoxycorticosterone, uniquely associated with %DMAs. Again, %DMA associations of metabolites tended to be in the opposite direction of the U-tAs association. The metabolites associated with arsenicals in non-diabetics could be related to metabolic processes related to the development of diabetes making them potential targets of interest for study in understanding how arsenic exposure leads to diabetes development.

Lastly, we compared the metabolomic fingerprint metabolites to those identified in association with *AS3MT* genotype in Chapter 3. We identified three of the fingerprint metabolites that were associated with one SNP in *AS3MT*. These were 5-aminolevulinic acid, which was associated with rs3740390 in the overall model. Oxalic acid was identified in association with rs17881215 in diabetic individuals alone. 11-eicosenoic acid was identified in association with

rs11191439 and rs17881215 in non-diabetic individuals alone. These metabolites may be related to risk of diabetes development thus playing a role in inter-individual susceptibility.

The comparison of the identified metabolites to those identified as an arsenic exposure-associated diabetes fingerprint reveals that arsenic metabolism and genotype of *AS3MT* may be related to development of arsenic-associated diabetes. If these things were unrelated we would expect that there would be no overlaps between the metabolite sets. The presence of these overlaps suggests that these metabolites may be targets for further study in understanding the relationship between arsenic exposure, metabolism and risk of diabetes development.

Arsenic metabolism and genotype for *AS3MT* are linked to alterations related to mechanism of arsenic-associated diabetes

Given that arsenic metabolism has been linked to disease outcomes, and that *AS3MT* genotype has been linked to development of arsenic metabolism, overlaps between metabolic profiles associated with arsenicals and *AS3MT* genotypes were of interest. Interestingly, we observed overlaps between the profiles. Among non-diabetic individuals, only the plasma metabolite 2,5-dihydroxybenzoic acid overlapped between arsenical profiles and allelic variation of SNP rs3740390 in non-diabetic individuals (Figure 8). It is a result of metabolism of acetyl salicylic acid (aspirin). Interestingly, increased production of this metabolite is associated with increased stress on the oxidative-stress response system and has been proposed as an early biomarker of oxidative stress in diabetic individuals [169]. It has also been shown that production of this plasma metabolite is higher in diabetic rats than non-diabetic rats and may be associated with diabetes-related complications[168]. We observed a decrease of this metabolite in association with increasing %iAs in non-diabetic individuals exposed to arsenic, as well as an increase in the 2,5-dihydroxybenzoic acid in relationship to the presence of the variant allele for

the SNP rs3740390. These data suggest that metabolism in carriers of the variant allele could be associated be experienced increased oxidative stress. Additionally, it also suggests that higher levels of %iAs may be an indicator of lower oxidative stress.

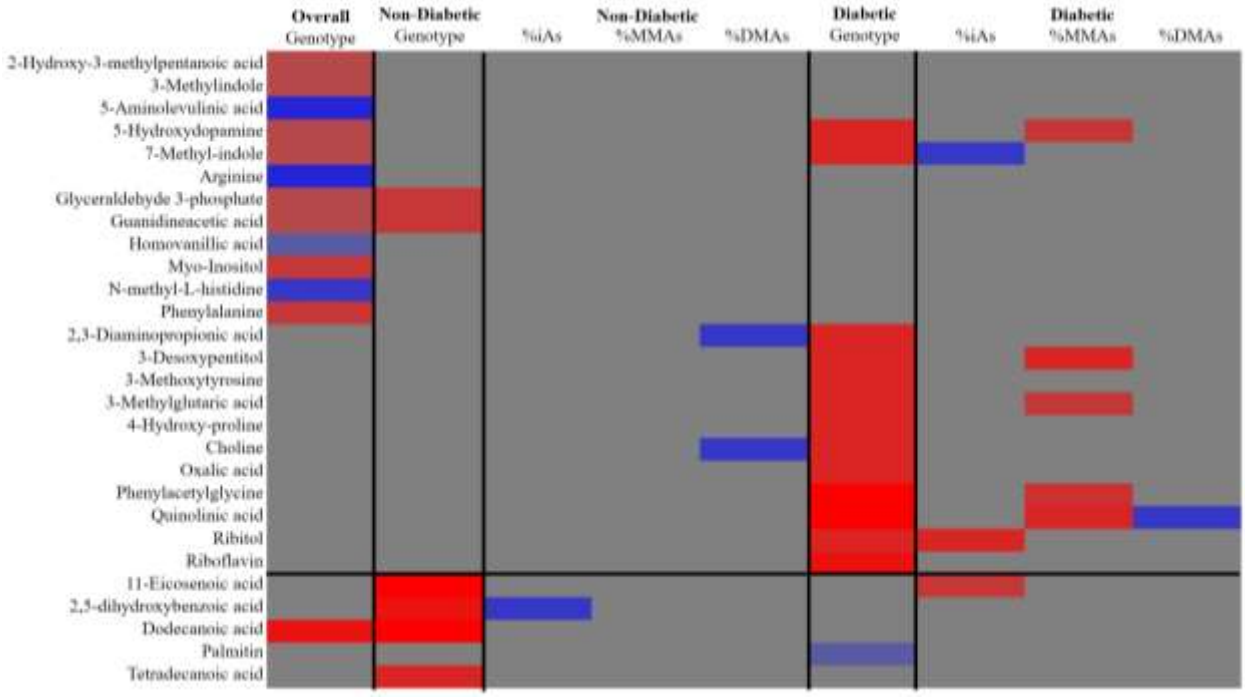


Figure 8. Comparison of metabolites associated with allelic variation in one of six AS3MT SNPs and arsenical profiles.

Also among non-diabetic individuals we observed decreased levels of the metabolites 2,3-diaminopropanoic acid and choline in association with %DMAs (Figure 8). Increased levels of these metabolites were associated with the presence of the variant alleles for SNP rs17881215 in diabetic individuals. At present there is little knowledge regarding the role of 2,3-diaminopropanoic acid in human health. However, choline is a methyl donor and essential nutrient in humans. Choline is involved in regulation of homocysteine concentrations. This is directly relevant to arsenic metabolism as homocysteine plays a key role in the regulation of the second methylation step that converts MMAs to DMAs. Furthermore, choline deficiency

attenuates insulin resistance and glucose intolerance in mice. Additionally, choline induces glucose and insulin resistance through increase glucagon action, i.e. promotion of gluconeogenesis. This suggests that arsenic-associated diabetes could be a combination of insulin resistance through increase gluconeogenesis. Individuals carrying the variant allele of rs1788125 could be predisposed to lower choline utilization efficiency, thus making them more likely to develop a diabetes phenotype.

Among diabetic individuals, we observed overlap between three metabolites associated with %iAs, and genotypic variation (Figure 8). The plasma metabolite 11-eicosenoic acid was associated with genotypic variation in the overall model in non-diabetic individuals. It was also associated with %iAs in diabetic individuals. This metabolite is a long chain unsaturated acid. Interestingly, it has been shown that incubation of insulin-producing cells with 11-eicosenoic acid is protective against lipotoxicity induced by fatty acids, specifically palmitin which was associated with genotype in diabetic individuals. This suggests that perhaps that individuals who carrier the variant allele for rs17881215 or rs.11191453 have higher levels of 11-eiconsenoic acid making their pancreatic beta cells less susceptible to toxicity. Additionally, as arsenic is associated with decreased lipid storage capacity of LT3 cells, it is possible that increased 11-eiconsenoic acid acts as a protective mechanism against arsenic induced lipotoxicity in the pancreas.

A total of five metabolites were associated with %MMAs and genotypic variation in both the diabetic individuals (Figure 8). These metabolites were: 5-hydroxydopamine, 3-desoxypentitol, 3-methylglutaric acid, phenylacetylglycine, and quinolinic acid. Interestingly quinolinic acid was decreased in association with increasing proportions of DMAs. Higher levels of 3-methylglutaric acid, phenylacetylglycine, and quinolinic acid have all be observed at higher

levels in healthy subjects as compared to their diabetic counterparts. In addition, quinolinic acid, a product of tryptophan metabolism, has been shown to directly inhibit phosphoenolpyruvate carboxykinase, resulting in hyperglycemia in rats. Given their association with increased %MMAs and variant alleles for SNPs previously associated with increased proportions of MMAs in urine it is possible that individuals are predisposed to generating increased levels of the metabolites and that arsenic metabolism increases these metabolites. These data suggest that there is perturbation of phenylalanine and tryptophan metabolism in response to arsenic metabolism resulting in increased susceptibility to diabetes. These metabolites represent a key area of focus for future research as they are likely important in the development of diabetes in the context of arsenic exposure.

Arsenic exposure, metabolism, and inter-individual differences are related to arsenic-associated diabetes through a series of metabolic pathways

The effects of arsenic exposure are dependent on metabolism of arsenic which is related to genotype of *AS3MT* (Figure 9). The metabolism of arsenic results in alterations of enzymatic process throughout the body (Figure 9). Both the metabolism of arsenic as well as the alterations of other enzymatic processes are directly related to β-cell dysfunction and alterations in insulin signaling processes that result in hyperglycemia (Figure 9).

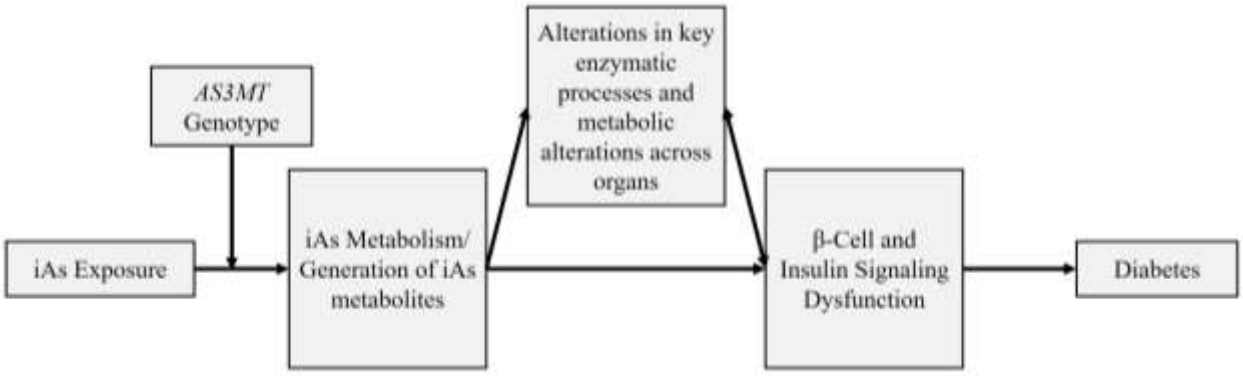


Figure 9. Integrative schematic describing the relationship between arsenic exposure, genotype of AS3MT, metabolism that lead to dysfunctional phenotypes of diabetes.

This research demonstrates that some of the processes that are either altered in response to arsenic exposure or in response to diabetes developed in the presence of arsenic are traditional diabetic processes. This is evidenced by alterations in metabolites that have been previously related to diabetes development in response to arsenic exposure and arsenic metabolism. These include the five branch chain amino acids that have been used as predictive biomarkers for diabetes. Given that these were observed as altered in response to arsenic exposure and arsenic metabolism, it is likely that arsenic works through traditional pathways for induction of diabetes. Additionally, alterations of the TCA cycle were observed consistently in both diabetic and non-diabetic individuals. Given the key role of the TCA cycle in the production of ATP and it's use of glucose, it seems likely that arsenic induces alterations in the TCA cycle that are perpetuated in the diabetic condition. Lastly, alterations in vitamin B metabolism were observed in response to arsenic exposure and metabolism. Given that vitamin B metabolism has been previously observed in response to diabetes and have been observed to attenuate arsenic-related disease previously, Vitamin B, specifically riboflavin, supplementation could represent a candidate for intervention studies. Taken together these data suggest that arsenic alters processes altered in

traditional diabetes and some of these processes could provide targets for intervention-based strategies to prevent diabetes development or prevent diabetic complications.

Additionally, the results of these studies provide evidence that arsenic-associated diabetes is also related to non-traditional pathways of diabetes development. This is clearly evidenced by the 59 metabolites related to U-tAs in diabetic individuals the majority of these metabolites n=47, 80% these metabolites have not been observed in other metabolomic studies of diabetic individuals. Furthermore, the research presented here suggests that a subset of these metabolites are related to alterations in arsenic metabolism and genotype. Some of these metabolites could provide insights into inter-individual susceptibility to diabetes development in the presence of arsenic exposure. They can also provide information on targets of arsenic toxicity as well as the role of metabolism in arsenic, associated disease. Taken together, these 47 metabolites provide novel targets for understanding the development of arsenic-associated diabetes.

Importantly, the collected studies provide evidence for mechanisms by which arsenic could potentially induce diabetes. The identification of quinolonic acid provides further support for the relationship between arsenic induction of gluconeogenesis. Additionally, 11-eiconsenoic acid and plamitin suggest lipotoxicity may be a mechanism by which arsenic is able to suppress insulin secretion and induce pancreatic β-cell apoptosis. 2,5- dihydroxybenzoic acid suggest the that oxidative stress response to arsenic likely plays a role in the development of diabetes. Lastly, the relationship between choline, arsenic metabolism and *AS3MT* genotype suggests a mechanism by which %DMAs are related to diabetes. Increased proportions of choline are linked to decreased proportions of homocysteine, and decreased proportions of homocysteine are linked to increased proportions of %DMAs. Increased choline levels are link associated with insulin resistance and induction of gluconeogenesis. These effects could provide a portion of the

explanation for the relationship between high %DMAs and development of diabetes. These data suggest that many of the metabolites identified could help provide mechanistic understanding of the relationship between arsenic exposure and diabetes.

Taken together these data provide insight into traditional diabetes processes altered by arsenic exposure, suggest that some processes related to diabetes development in the context of arsenic exposure are unique to the condition and that metabolomics can provide a tool for identifying mechanistic pathways by which arsenic, arsenic metabolism and genotype relate to susceptibility to diabetes development. Additionally, these collected provide strong support for the use of metabolomics as an important tool for mechanistic understanding in humans.

Public Health Relevance

Globally, more than 200 million people are exposed to inorganic arsenic at levels that far exceed the EPA and WHO standard. Our research examining the relationship between metabolomic profiles of diabetic and non-diabetic individuals exposed to arsenic reveals potential mechanisms by which arsenic may induce diabetes in humans. Importantly, it also reveals the relationship between arsenic, metabolic pathways including vitamin B metabolism, amino acid metabolism and energy metabolism, and diabetes development. These pathways, particularly riboflavin related pathways, serve as potential targets for population-based intervention strategies. Nutrient supplementation of arsenic-exposed populations has shown success previously in mitigating disease development and our research points towards potential pathways of interest that might be relevant to prevention of diabetes development. Additionally, our findings demonstrate that arsenic may alter pathways traditionally altered in type 2 diabetes making some of the newly developed biomarkers, specifically the five branched chain and

aromatic amino acids, effective for early identification of susceptibility individuals. Lastly, we demonstrate the potential mechanistic relationship for a gene by environment by disease interaction. Specifically, we show that genotypes of *AS3MT* are related to metabolites that are known to be altered in diabetes. A set of these metabolites are also altered in response to arsenic exposure and arsenic metabolism. These findings can help our understanding of inter-individual difference which in turn allows for better protections of susceptible populations. Furthermore, this research is directly relevant to human subjects and can serve as a model for future human population based mechanistic work. We have effectively utilized an untargeted metabolomic profiling approach to identify metabolites altered in association with arsenic-related and disease-related processes. Additionally, this work was conducted in readily obtainable bio specimens, demonstrating the broad applicability of metabolomics to many fields of studies. The results demonstrate that the integration of metabolomic data with other data types, such as existing information of toxicant metabolism, and genotypic data is a viable technique for understanding mechanisms of disease development as well as underlying mechanisms of inter-individual susceptibility. Taken together these studies provide the basis for future hypothesis for prevention and treatment, adds to the existing body of literature about health effects of arsenic exposure that can be used for regulatory purposes, as well as demonstrate the relevance of metabolomic profiling in public health research.

Relationship of Current Research to Future Hypotheses

Our research reveals metabolic pathways that are mechanistically associated with arsenic exposure and diabetes development. This is among the first studies to apply the use of metabolomic profiling in the context of environment-disease research. For this reason, there are clear areas to target to improve the mechanistic understandings and public health relevance

garnered from this study. Our findings provide the basis for hypothesis generation and research questions, include those detailed here.

1. **What enzymes are altered in association with metabolites associated with arsenic-associated diabetes?**

Results from our study suggest that arsenic exposure alters enzymatic activity related to processes involved in diabetes development. However, it is currently unknown which precise enzymatic processes, tissues and organ systems are responsible for the observed impacts. Given that urinary metabolites represent many diverse organ systems, i.e. metabolites in urine may be derived from the blood stream by the kidneys, they could be impacted by organ-based arsenic-associated alterations. Identifying the target sites at which these metabolites are produced and which enzymes are affected is critical to understanding which processes are associated with arsenic-induced diabetes.

A major strength of cell culture work is it is performed directly in the cell type of interest, therefore enzymes can be inhibited at to understand whether that is the controlling factor in the observed phenotype. However, at present cell culture cannot link multiple organ systems together as they would be in a complete organism. While only indirect measures of the metabolites are possible in human population research, one of its major strengths as the ability to observe the whole system working together. Working towards understanding what enzymes are targeted by arsenic exposure could provide insight into which organ systems are altered and thus provide context for the metabolic alterations that have occurred. This could help focus the field not those metabolites that would be crucial for development of intervention and therapeutic strategies. Furthermore, the understanding of arsenic's enzymatic targets in the cell could provide mechanistic understanding for its multimodal effects, not just those related to diabetes.

The hypothesis that could be tested would be: Enzymes related to arsenic-associated diabetes would be those know to alter the levels of the metabolites identified in urine.

Computational methods provide the best hope for understanding enzymatic processes tied to metabolomic profiles. The development of a comprehensive database of metabolites, their relationships to enzymatic processes and which organ systems they exist. At present these data have to be manually curated, however using machine learning and integrating a use interface could be useful. Additionally, a computational model would have the added benefit of being able to integrate across cell culture and human population models. As more metabolomics studies are conducted, the need for this type of database will get strong, and the need for this type of program would be invaluable to numerous researchers.

2. What nutritional modifiers could be used for intervention in human population studies?

Given the public health focus of this research it is important to think about how the present research could result in intervention with the goal of disease prevention. The present work has focused on identification of what pathways are linked to arsenic-associated diabetes. Among these are choline and vitamin B. In previous research it has been shown that arsenic metabolism can be shifted to produce more DMAs and less MMAs by supplementation with folate. Additionally, researchers have focused on understanding the relationship between numerous anti-oxidants as to prevent arsenic related disease. Finally, vitamin B has been associated with attenuated disease risk in the context of arsenic exposure, specifically for skin lesions. Given this successful history of supplementation, understanding which pathways can be targeted to decrease the risk of diabetes development and testing of these trials through intervention based strategies could mitigate disease burdens in countries with highly exposed

populations. The hypothesis that could be tested would be: Riboflavin can prevent arsenic-induced effects in cell culture, and riboflavin supplementation of an arsenic exposed population will decrease the number of individuals who develop diabetes.

To make intervention possible, one would have to tests each of the four mechanisms by which arsenic is able to induce diabetes and the nutritional modifier. Given the results of the present research, riboflavin would be a good modifier to test first. One could set up cell culture models like those described in the introduction, to test the effects of riboflavin on (i) insulin resistance/signaling, (ii) pancreatic β-cell damage, (iii) pancreatic β-cell dysfunction, and (iv) induction of gluconeogenesis. If riboflavin was successful at preventing arsenic and its metabolites from inducing these effects in cell culture, animal models could be tested next and then it could be applied to human populations.

3. What is the mechanistic role of *AS3MT* in inter-individual differences on disease risk?

Given that AS3MT is the major enzyme responsible for arsenic metabolism, it is obvious that it would play a role in disease susceptibility related to arsenic exposure. Interestingly the majority of these variants are found in non-coding regions of the gene. This suggests that the causal linkage between genotype and the observed effects is either related to regulatory capacities of region. In fact, it has been previously linked to proportions of arsenic metabolites in urine as well as odds of an individual being diabetic. Our work elucidates metabolites linked to differences in *AS3MT* genotype. Understanding what enzymes and processes these metabolites represent is crucial for understanding how *AS3MT* genotype influences disease susceptibility. The hypothesis to be tested would be: Diverse cell culture and animal models (i.e. those with variations in *AS3MT*) will display different metabolic alterations in response to arsenic exposure.

To better understand the relationship between *AS3MT* genotype and disease susceptibility, future research could include cell culture models and/or genetically diverse animal models. One experiment would be to examine the response of diverse cell lines to arsenic exposure to determine differences in enzymatic and metabolic alterations. The other would be to expose genetically diverse animal models to arsenic and use metabolomic profiling of urine and serum as well as target tissue assessment to understand differences in *AS3MT* expression and other enzymatic activity on disease susceptibility and progression.

Conclusions

In summary, a major contribution of this work is the finding of metabolomic profiles that differentiate arsenic-associated diabetes from traditional type II diabetes. This is demonstrated by the differences in metabolomic profiles related to arsenic exposure, arsenic metabolism and inter-individual differences related to arsenic metabolism (*AS3MT* genotype). Among these are metabolites that play critical roles in processes of energy metabolism, amino acid metabolism and vitamin B metabolism. Collectively, these studies increase knowledge related to the mechanisms by which arsenic-induces diabetes in humans, acting as a foundation for future toxicology and public health research.

APPENDIX 1

Supplemental Table 1. Studies detailing the relationship between arsenic exposure and diabetes development in both highly exposed (median >150 µg iAs/Lin drinking water) and low exposure (median <150 µg iAs/L in drinking water).

Study Design	Location	Diabetes Diagnosis	Exposure Measure (mean)	Analysis Factors	Major Finding
			High Exposure		
Chen et al 2010 (cross-sectional)	Bangladesh, HEALs (n=11,319)	Self-reported prior to baseline	DW-iAs, U-tAs	Age, sex BMI, smoking status, education	adjOR=1.11 (0.73-1.69)
Chiu et al 2006 (ecological)	Taiwan (n=139,051)	Death Certificate	Drinking Water Supply Change	Unadjusted	Females SMR= 1.53 (p<0.01)
Huang et al 2014 (cross-sectional)	Cambodia (n=142)	HbA1c, Blood glucose level	DW-iAs, U-tAs, arsenicosis	Unadjusted	DW-iAs adjOR= 1.7 (0.5-5.8), arsenicosis adjOR=1.7 (0.5-5.6)
Lai et al 1994 (cross-sectional)	Taiwan (n=891)	Self-reported, OGTT, treatment history	CEI (mean and range)	Age, sex, BMI, physical activity	adjOR=10.005 (1.3-77.9)
Nabi et al 2005 (cross-sectional)	Bangladesh (n=235)	Blood Glucose	DW-iAs	Unadjusted	OR=2.95(0.954 -9.279)
Rahman et al 1999 (cross-sectional)	Bangladesh (n=1,107)	Self-reported, OGTT, glycosuria	Keratosis vs. non-karatosis	Age	adjPR=5.2 (2.5-1.5)
Rahman et al 1998 (cross-sectional)	Bangladesh (n=430)	Glycosuria	DW-iAs, CEI	Age, sex, BMI	adjPR=2.9 (1.6-5.2)
Tsai et al 1999 (retrospective)	Taiwan (n=19,563 deaths)	Death Certificate	Endemic regions vs. national reference	Age, sex	SMR=1.46 (1.28-1.67)
Tseng et al 2000a, 2000b (prospective cohort)	Taiwan (n=446)	Fasting blood glucose, OGTT	CEI, artisan well	Age, sex, BMI	RR=2.1 (1.1-4.2)

Wang et al 2003 (cross sectional)	Taiwan (n=706,314)	Insurance Claims	Endemic vs. non-endemic regions	Age, sex	adjOR=2.69 (2.65-2.73)
			Low Exposures		
Brauner et al 2014 (prospective cohort)	Denmark (n=57,053)	Diabetes discharge diagnosis, treatment history/purchases	Time weighted arsenic exposure (calculated)	adjusted for age, BMI, sex, waist circumference, smoking, environmental tobacco smoke, dietary intake, education, SES	Adjusted IRR=1.03 (1.01-1.06)
Coronado-Gonzales et al 2007 (case control)	Mexico (n=400)	Fasting blood glucose, treatment history	U-tAs (creatinine adjusted), DW-iAs	Age, sex, hypertensions, family history, obesity, serum lipids	adjOR=2.84 (1.64-4.92)
Currier et al 2014 (cross-sectional)	Mexico (n=374)	Fasting blood glucose, OGTT, self-reported, use of medication	exfoliated urothelial cells	Age, sex, BMI	adjOR iAs= 1.57 (1.19-2.07), MMAs=1.63 (1.24-2.15) , DMAs=1.31 (0.96-1.84)
Del Razo et al. 2011 (cross-sectional)	Mexico (n=258)	Fasting blood glucose	DW-iAs	Age, sex, obesity, hypertension	adjOR=1.13 (1.05-1.22) per 10 µg As/L
D'lppoliti et al 2015 (cross-sectional)	Italy (n=165,609)	Mortality Registry	Cumulative arsenic does indicator (calculated)	Age, calendar period, employment at ceramics industry	Cox Hazard Ratio=2.56 in females
Ettinger et al 2009 (cross-sectional)	USA (n=456 pregnant females)	Impaired glucose tolerance (OGTT)	Blood iAs, Hair iAs	Age, pre-pregnancy BMI, race, medicated use, married/living with partner	adjOR=2.79 (1.13-6.87)
Ettinger et al 2014 (cross-sectional)	USA (n=150 African decent young adults)	Elevated fasting blood glucose (not diabetes)	Blood iAs	recruitment site, employment status, education, marital status, smoking, alcohol, fish intake, age, sex	adjOR=4.1 (1.2-14.6)
Feng et al 2015 (cross-sectional)	China (n=2242)	Fasting plasma glucose	U-tAs	Age, BMI, sex, hypertension, hyperlipidemia, family history of diabetes, anti-diabetes drug/insulin use	adjOR=1.827 (1.096-3.045)

Feseke et al 2015 (cross-sectional)	Canada (n=3151)	Blood glucose, HbA1c	U-tAs	Age, sex, educational level, alcohol, smoking, BMI, hypertension, creatinine	Diabetes adjOR=1.81 (1.12-2.95) Prediabetes adjOR= 2.04 (1.03-4.05)
Gribble et al 2012 (cross-sectional)	USA (n=3924)	Fasting plasma glucose, OGTT, HbA1c or diabetes medication	Baseline U-tAs	Age, sex, urinary creatinine, education, smoking alcohol, BMI	adjOR= 1.55 (1.39-1.73)
Islam et al 2012 (cross-sectional)	Bangladesh (n=1004)	Fasting blood glucose, self-reported	CEI via drinking water	Age, sex, education, BMI, family history of diabetes	RR=1.9 (1.1-3.5)
James et al 2013 (cohort)	USA (n=1096)	Fasting plasma glucose, OGTT, self-reported with verification	Time-weighted average in residential drinking water	Race, BMI, physical activity	adjOR= 1.27 (1.01-1.59)
Janovic et al 2013 (cross-sectional, ecological)	Serbia: Exposed region (n=195,190) n vs. general population(n =1,324,489)	National registry of diabetes	Public water supply system iAs levels	Unadjusted	OR=1.32 (1.01-1.73)
Kim et al 2011 (cross-sectional)	South Korea, NHANES (n=1,677)	Fasting blood glucose, self-reported, or diabetes medication	U-tAs, creatinine adjusted	Age, sex, BMI, smoking, alcohol, education, hypertension, region, urban/rural, seafood consumption	RR=1.31 (1.04-1.66)
Kim et al 2013 (case-control)	USA (n=900)	OGTT	Baseline U-tAs	Age, sex, BMI, creatinine	1.11 (0.7-1.57)
Kuo et al 2015 (prospective cohort)	USA (n=1694)	Blood glucose, self-reported diabetes history or	U-tAs	Sex, education, smoking status, alcohol, BMI, waist to hip ratio, urinary creatinine	Higher %MMA is associated with lower risk of diabetes HR=0.84 (0.76-0.94)

		medication use			
Lee et al 2013 (cross-sectional)	South Korea, NHANES (n=3,393)	Blood glucose, anti-diabetic medication, self-reported	U-tAs	Sex, age, group, residence location, season, educational level, smoking status, drinking status, BMI, physical activity, seafood consumption	adjOR=1.654 (1.03-1.518)
Lewis et al 1999 (retrospective)	USA (n=961 male deaths, 1242 female deaths)	Death certificate	DW-iAs (well water)	Sex, race	Females: SMR= 1.23 (0.86-1.71) Males: SMR= 0.79 (0.48-1.22)
Li et al 2013 (cross-sectional)	China (n= 669)	Fasting plasma glucose, or use of diabetes medication	DW-iAs	Age, Sex, BMI, alcohol, smoking, cumulative arsenic exposure	adjOR=1.58 (0.58-4.26)
Mahram et al 2013 (ecological)	Iran (n=15,069)	Fasting plasma glucose	Endemic vs. non-endemic regions	Unadjusted, stratified by sex	adjOR= 1.03 (1.02-1.04)
Makris et al 2012 (cross-sectional)	Cyprus (n=317)	Self-reported	CEI estimated via regional data	Age, sex, smoking	adjOR= 1.86(0.30-11.59)
Meliker et al 2007 (retrospective)	USA (n=41,282 male deaths, n=38,722 female deaths)	Death certificate	DW-iAs	Sex, race	Females: SMR= 1.28 (1.18-1.37) Males: SMR= 1.27 (1.19-1.35)
Mendez et al 2016 (cross-sectional)	Mexico (n=1,160)	Blood glucose levels	DW-iAs, U-tAs	Age, sex, education, ethnicity, smoking, alcohol, waist circumference, BMI, primary source of drinking water, seafood intake	DW-iAs adjOR= 1.65 (0.97-2.81), U-tAs adjOR=1.99 (1.19-3.33)
Navas-Acien et al 2008 (cross-sectional)	USA, NHANES (n=788)	Fasting blood glucose, self-reported, medication	U-tAs	Sex, age, race, creatinine, education, BMI, serum cotinine, hypertension medication, urine arseno-betaine, blood mercury	adjOR=3.58 (1.18-10.83)

Navas-Acien et al 2009 (cross-sectional)	USA, NHANES (n=1279)	Fasting blood glucose, self-reported, medication	U-tAs	Sex, age, race, creatinine, education, BMI, serum cotinine, hypertension medication, blood mercury	adjOR=2.60 (1.12-6.03)
Pan et al 2013 (cross-sectional)	Bangladesh (n=957)	HbA1c	DW-iAs, toenail arsenic	Cigarette smoke, BMI, age, sex, status of skin lesions	adjOR=4.51 (2.01-10.09)
Peng et al 2015 (retrospective case control)	China (n=431)	Antenatal care records	U-tAs	Newborn sex, maternal age, pre-pregnancy BMI, gravidity and parity	adjOR= 5.25 (1.99-13.86) (1st vs. 4th)
Peng et al 2015 (cross-sectional)	USA, NHANES (n=835)	Fasting glucose, insulin	U-tAs, iAs, DMAs	creatinine, sociodemographic factors, BMI, waist circumference, arseno-betaine	adjOR= 0.25% (0.23- 0.29) associated with HOMA-IR
Rhee et al 2013 (cross-sectional)	South Korea, NHANES (n=3,602)	Fasting blood glucose, Serum insulin	U-tAs, creatinine adjusted	BMI, physical activity, educational status	adjOR=1.56 (1.03-2.36)
Ruiz Navarro et al 1998 (case-control)	Spain (n=87 hospital patients)	Not reported	U-tAs	Unadjusted	RR=0.87 (0.5-1.53)
Shapiro et al 2015 (cross-sectional)	Canada (n=1274 pregnant women)	Impaired glucose tolerance and gestational diabetes in chart	U-tAs	Age, race, pre-pregnancy BMI, education	adjOR= 3.7 (1.4-9.6)
Steinmaus et al 2009a (cross-sectional)	USA, NHANES (n=795)	Fasting blood glucose, self-reported, medication	U-tAs	Sex, age, race, creatinine, education, BMI, serum cotinine, hypertension medication, blood mercury	adjOR=1.15 (0.53-2.50)
Steinmaus et al 2009b (cross-sectional)	USA, NHANES (n=1280)	Fasting blood glucose, self-reported, medication	U-tAs	Sex, age, race, creatinine, education, BMI, serum cotinine, hypertension medication	adjOR=1.03 (0.38-2.80)
Tollestrup et al 2003	USA (n=1074 deaths)	Death certificate	Residence time within an	Unadjusted	RR=1.6 (0.36-7.16)

(retrospective)			arsenic area		
Wang et al 2007 (cross-sectional)	Taiwan (n=660)	Metabolic Syndrome (Fasting Plasma Glucose)	High vs. low iAs in hair	Age, sex, occupation, lifestyle factors	adjOR=2.53 (1.02-5.43)
Wang et al 2009 (cross-sectional)	China (n=235)	Hospital records, exam	DW-iAs	Unadjusted	RR=1.098 (0.98-1.231)
Ward and Pim 1984 (cross-sectional)	UK (n=117 diabetes clinic patients)	Not Reported	Blood iAs	Unadjusted	RR=1.09 (0.79-1.49)
Zierold et al 2004 (cross-sectional)	USA (n=1185 well water testing participants)	Self-reported	DW-iAs	Age, sex, BMI, smoking	adjOR=1.02 (0.49-2.15)

*OR=odds ratio
*adjOR=adjusted odds ratio
*PR=prevalence ratio
*RR=relative risk
DW-iAs= drinking water inorganic arsenic
U-tAs= Urinary total arsenic (sum of iAs, MMAs, DMAs)
IRR=incidence risk ratio

APPENDIX 2

Supplemental Table 2. Metabolites identified with altered levels (either positive or negative) associated with U-tAs in either urine (U) or plasma (P) in diabetic (D) or non-diabetic (ND) individuals and their respective beta values.

HMDB	Metabolite	Urine	Plasma	Urine ND	Urine D	Plasma ND	Plasma D	Comparison to previous studies
HMDB 02453	**(R*,R*)-2,3-Dihydroxybutanoic acid**		(-) PD				-0.221502	
HMDB 01961	**1,7-Dimethylguanosine**	(+) UD			0.208906			
HMDB 10351	**11-beta-hydroxyandrosterone-3-glucuronide**	(+) UND, UD		0.254513	0.232284			
HMDB 02231	**11-Eicosenoic acid**		(+) PD				0.207193	
HMDB 10338	**11-Oxo-androsterone glucuronide**	(+) UD			0.325399			
HMDB 03099	**1-Methyluric acid**	(+) UND		0.238989				
HMDB 00370	**2-Amino-3-phosphonopropionic acid**	(-) UND, UD		-0.399353	-0.397866			
HMDB 00337	**2-Deoxytetronic acid**		(+) PD				0.225864	
-	**2-furanylmethanol**	(-) UND, UD		-0.32544	-0.279091			
HMDB 00317	**2-Hydroxy-3-methylpentanoic acid**	(+) UD			0.209135			
HMDB 00321	**2-Hydroxyadipic acid**	(+) UD			0.237492			
HMDB 00606	**2-Hydroxyglutaric acid**	(+) UND		0.283882				
HMDB 02285	**2-Indolecarboxylic acid**	(+) UD			0.452335			
HMDB 00379	**2-Methylcitric acid**	(+) UND, UD		0.247932	0.347649			

HMDB 00426	**2-Methylmalic acid**	(+) UND		0.24978				
HMDB 02176	**2-Methyloctanoate**	(+) UD			0.205282			
-	**2'-Nitro-4',5'-dimethoxyacetophenone**	(+) UND		0.376147				
HMDB 00392	**2-Octenoic acid**	(-) UND		-0.256751				
HMDB 02441	**3,3-Dimethylglutaric acid**	(+) UD			0.280389			
-	**3,4-Dehydro-DL-proline**		(-) PD				-0.202722	
HMDB 00323	**3-Amino-2-piperidone**		(-) PD				-0.27569	
HMDB 03911	**3-Aminoisobutanoic acid**	(+) UD			0.23779			
HMDB 01885	**3-Chlorotyrosine**	(-) UND, UD		-0.431965	-0.3483			
HMDB 00346	**3-Deoxyarabinohexonic acid**		(+) PD				0.203775	
HMDB 00355	**3-hydroxy-3-methylglutaric acid**	(+) UD			0.263935			Kaur
HMDB 01476	**3-Hydroxyanthranilic acid**	(+) UND		0.215583				
HMDB 00413	**3-Hydroxydodecanedioic acid**	(+) UD			0.363839			
HMDB 06116	**3-Hydroxyhippuric acid**		(-) PND			-0.238873		
HMDB 00496	**3-Methoxy-4-hydroxyphenylglycol glucuronide**	(+) UD			0.345336			
HMDB 03332	**3-Methoxy-4-Hydroxyphenylglycol sulfate**	(+) UD			0.330573			
HMDB 03771	**3-Methyl-2-ketobutyric acid**		(+) PND			0.236812		
HMDB 00459	**3-Methylcrotonylglycine**	(+) UD			0.36697			

HMDB 00555	**3-methyl-hexanedioic acid**	(+) UD			0.230153	
HMDB 01527	**3-Methylthiopropionic acid**		(-) PND			-0.312448
HMDB 04813	**3-Methyluridine**	(+) UND, UD		0.471367	0.352614	
HMDB 01867	**4-Aminohippuric acid**	(-) UND		-0.23088		
HMDB 00500	**4-Hydroxybenzoic acid**	(-) UND		-0.241778		
HMDB 01988	**4-Hydroxycyclohexylcarboxylic acid**	(-) UD			-0.262586	
HMDB 02040	**4-Methoxycinnamic acid**		(-) PND			-0.236579
HMDB 00695	**4-Methyl-2-Oxovaleric acid**	(-) UD			-0.201484	
HMDB 04058	**5,6-Dihydroxyindole**	(-) UND		-0.212518		
HMDB 01149	**5-Aminolevulinic acid**	(-) UD			-0.267897	
HMDB 04185	**5-Hydroxyindoleacetylglycine**	(-) UND, UD		-0.215543	-0.213705	
HMDB 02894	**5-Methylcytosine**	(+) UD			0.273109	
HMDB 00884	**5-Methyluridine**	(+) UND, UD		0.385381	0.207552	
HMDB 00267	**5-Oxoproline**	(+) UND		0.261195		
HMDB 01182	**6,8-Dihydroxypurine**	(-) UND		-0.323023		
HMDB 00897	**7-Methylguanine**	(+) UND, UD		0.260051	0.219655	
HMDB 10715	**Acetamide**	(-) UND, UD		-0.296614	-0.256844	
HMDB 00532	**Acetylglycine**	(+) UD			0.262967	

HMDB 01494	**Acetylphosphate**	(-) UND, UD		-0.363901	-0.395307			
HMDB 00034	**Adenine**	(+) UD			0.227012			
HMDB 00050	**Adenosine**	(+) UND, UD		0.230834	0.224235			
HMDB 02829	**Androsterone glucuronide**	(+) UND		0.19766				
HMDB 00568	**Arabitol**	(+) UND, UD		0.350478	0.242236			
HMDB 06483	**Aspartic acid**	(-) UND, UD		-0.243953	-0.337276			
HMDB 00030	**Biotin**	(+) UD			0.400786			
METPA 0048	**Carnitine**	(-) UD			-0.227683			Mihlik
HMDB 00072	**cis-Aconitic acid**	(+) UND, UD		0.344395	0.230832			
HMDB 00904	**Citrulline**		(-) PD				-0.296527	Zhou
HMDB 00063	**Cortisol**		(-) PD				-0.251697	
HMDB 00562	**Creatinine**	(-) UND		-0.243142				
HMDB 00058	**Cyclic AMP**	(+) UND, UD		0.242615	0.371931			
HMDB 00192	**Cystine**	(+) UND		0.237605				
HMDB 00450	**Delta-Hydroxylysine**	(-) UD			-0.235906			Zhou
HMDB 00016	**Deoxycorticosterone**	(-) UD	(-) PND		-0.421421	-0.322224		
HMDB 01321	**Erythrose 4-phosphate**		(-) PND			-0.209521		
HMDB 01786	**Ethenodeoxyadenosine**		(+) PND			0.498351		
HMDB 02023	**Ethyladipic acid**		(-) PND			-0.28906		
METPA 0178	**Ethylene glycol**	(-) UND, UD		-0.344224	-0.231918			
HMDB 00134	**Fumarate**		(+) PD				0.192405	
HMDB 00107	**Galactitol**	(+) UND, UD		0.307447	0.214455			

HMDB 00565	**Galactonic acid**	(+) UND, UD		0.253801	0.234901			
HMDB 00663	**Glucaric acid**	(+) UND		0.373981				
-	**Glucoheptose**	(+) UND		0.297107				
HMDB 00150	**Gluconic acid, lactone**		(+) PD				0.213474	
HMDB 00620	**Glutaconic acid**	(+) UD			0.292332			
HMDB 00641	**Glutamine**	(-) UND		-0.249229				
HMDB 00661	**Glutaric acid**	(+) UD			0.214054			
-	**Gluticol**	(+) UD			0.204915			
HMDB 01112	**Glyceraldehyde 3-phosphate**	(-) UND		-0.316253				
HMDB 00131	**Glycerol**	(-) UD			-0.217148			
HMDB 00123	**Glycine**		(+) PD				0.220799	Gall, Mennesa, Zhou, Zhang, Thalacker, Geidenstam
HMDB 00128	**Guanidineacetic acid**	(-) UND		-0.293315				
HMDB 00448	**Hexanedioic acid**	(+) UD			0.272758			
HMDB 03518	**Homocitric acid**	(+) UD			0.278779			
HMDB 01212	**Hydantoin-5-propionic acid**		(-) PND			-0.252515		
HMDB 02434	**Hydroquinone**	(+) UND		0.202069				
HMDB 00115	**Hydroxyacetic acid**		(-) PND			-0.221321		
HMDB 00193	**Isocitrate**	(+) UND		0.268509				
HMDB 00333	**Isohomovanillic acid**	(+) UD			0.21426			
HMDB 02092	**Itaconic acid**	(-) UND		-0.315275				Kaur
HMDB 00751	**L-Threo-2-pentulose**		(-) PND			-0.258552		
HMDB 00182	**Lysine**		(-) PD				-0.248871	Zhang
HMDB 10382	**LysoPC(16:0)**		(-) PND			-0.222764		

HMDB 00744	**Malate**	(+) UND, UD	(+) PD	0.276093	0.274521		0.229864	Manni
HMDB 00765	**Mannitol**	(+) UND, UD		0.29004	0.299893			
HMDB 00696	**Methionine**	(+) UD			0.321013			Zhang
HMDB 02108	**Methylcysteine**	(-) UND, UD		-0.311451	-0.267529			
HMDB 06471	**Methylisocitric acid**	(+) UD			0.278779			
HMDB 01844	**Methylsuccinic acid**	(+) UD			0.216871			
HMDB 02120	**Monoethyl phthalate**		(-) PND			-0.319714		
HMDB 00812	**N-Acetyl-L-aspartic acid**	(+) UND, UD		0.308397	0.213989			
HMDB 02201	**N-Carboxyethyl-g-aminobutyric acid**	(+) UD			0.214323			
-	**N-methylacetamide**		(+) PND			0.356132		
HMDB 00847	**Nonanoic acid**		(-) PND			-0.268792		
HMDB 02329	**Oxalic acid**	(-) UD			-0.220057			
HMDB 00223	**Oxaloacetic acid**	(-) UND		-0.334273				
HMDB 01587	**Phenylglyoxylic acid**	(+) UD			0.223359			
HMDB 00205	**Phenylpyruvic acid**	(-) UND, UD		-0.316189	-0.206255			
HMDB 00272	**Phosphoserine**	(-) UND		-0.339303				
HMDB 00822	**p-Hydroxymandelic acid**	(+) UND		0.269516				
HMDB 00162	**Proline**		(+) PD				0.264857	Zhou, Menni
HMDB 00767	**Pseudouridine**	(+) UND, UD		0.369608	0.243171			
HMDB 04230	**Pyrrole-2-carboxylic acid**	(-) UND, UD		-0.351292	-0.257609			
HMDB 00508	**Ribitol**	(+) UND, UD		0.229327	0.214792			

HMDB 01489	**Ribofuranose**	(+) UND	(+) PD	0.360752		0.214336		
HMDB 00867	**Ribonic acid**	(+) UND		0.222287				
HMDB 01900	**Ribono-1,4-lactone**	(+) UND, UD		0.33001	0.21305			
HMDB 00283	**Ribose**	(+) UND, UD		0.420205	0.301358			
HMDB 00187	**Serine**	(+) UD			0.228121			
HMDB 01266	**Sorbose**	(+) UD			0.233521			
HMDB 00252	**Sphingosine**		(-) PD			-0.243592		
HMDB 00254	**Succinate**	(+) UD			0.218772			Kaur
HMDB 00912	**Succinyladenosine**	(+) UND, UD		0.283036	0.297605			
HMDB 00258	**Sucrose**	(+) UND		0.22199				
HMDB 00251	**Taurine**	(-) UD			-0.227299			Kaur, Zhou
HMDB 04136	**Threitol**	(+) UD			0.301824			
HMDB 00925	**Trimethylamine N-oxide**	(-) UND		-0.266029				
HMDB 00158	**Tyrosine**		(-) PD			-0.203493		Wang, Zhang
HMDB 00289	**Uric acid**	(-) UND, UD		-0.350241	-0.293738			Kaur
HMDB 00301	**Urocanic acid**	(-) UND		-0.251512				

APPENDIX 3

Supplemental Table 3. Statistically significant associations for all metabolites associated with at least one arsenical measure in either diabetic or non-diabetic individuals.

	Non-Diabetic Individuals				Diabetic Individuals			
Urinary Metabolites	**U-tAs Associated**	**%iAs Associated**	**%MAs Associated**	**%DMAs Associated**	**U-tAs Associated**	**%iAs Associated**	**%MAs Associated**	**%DMAs Associated**
1,2-Benzenediol			0.235517					
11-beta-hydroxyandrosterone-3-glucuronide	Martin et al 2015 (Supp Table 2) (Supp Table 2)				Martin et al 2015 (Supp Table 2)		0.20384	
2,3-Diaminopropionic acid				-0.220947				
2,3-Dihydroxyvaleric acid						-0.205899		
2-Furoylglycine						-0.212067		0.203785
2-Hydroxy-2-methylbutyric acid			-0.218749	0.25416				
2-Hydroxybutyric acid			-0.280179	0.266319				
2-Hydroxycaproic acid						0.217334	-0.221035	
2-Indolecarboxylic acid					Martin et al 2015 (Supp Table 2)	0.237975		
2-Methoxyphenol							0.253132	
2-Methylmalic acid	Martin et al 2015 (Supp Table 2)						0.237801	
2'-Nitro-4',5'-dimethoxyacetophenone	Martin et al 2015 (Supp Table 2)	0.265042		-0.235846				
2-Octenoic acid	Martin et al 2015 (Supp Table 2)	-0.255561						
3,3-Dimethylglutaric acid					Martin et al 2015 (Supp Table 2)	0.376213		-0.316575
3,4,5-Trihydroxypentanoic acid							0.332187	

3-Desoxypentitol							0.288659	
3-hydroxy-3-methylglutaric acid					Martin et al 2015 (Supp Table 2)	0.22477		-0.231461
3-Hydroxyanthranilic acid	Martin et al 2015 (Supp Table 2)	0.225756		-0.201986				
3-Hydroxyphenylacetic acid			-0.23509	0.217605				
3-Methoxy-4-hydroxyphenylglycol glucuronide					Martin et al 2015 (Supp Table 2)	0.247291		
3-Methyl-3-hydroxybutanoic acid		-0.269186		0.292451				
3-Methylcrotonylglycine		0.218632			Martin et al 2015 (Supp Table 2)	0.236724		
3-Methylglutaric acid							0.234993	
3-Pyridylacetic acid			-0.231144	0.250417				
4-(3-Pyridyl)-3-butenoic acid						0.220707		
4-Aminohippuric acid	Martin et al 2015 (Supp Table 2)	-0.287061		0.278682				
4-Hydroxyphenylpyruvic acid							-0.21771	
5-Hydroxydopamine							0.228201	
5-Methylcytosine		0.21236		-0.210901	Martin et al 2015 (Supp Table 2)			
5-Oxoproline	Martin et al 2015 (Supp Table 2)						0.234926	
7-Methyl-indole						-0.209746		
Acetylphosphate	Martin et al 2015 (Supp Table 2)				Martin et al 2015 (Supp Table 2)			0.243668
Adenine					Martin et al 2015	0.217314		

					(Supp Table 2)			
Alpha-Hydroxyisobutyric acid				0.213239				
Alpha-N-Phenylacetyl-L-glutamine		-0.203687						
alpha-oxobenzeneacetic acid							0.257525	
Arabitol	Martin et al 2015 (Supp Table 2)				Martin et al 2015 (Supp Table 2)		0.292595	-0.248186
Asparagine		-0.218912		0.213148				
Aspartic acid	Martin et al 2015 (Supp Table 2)		-0.260472	0.251807	Martin et al 2015 (Supp Table 2)	-0.225884		
Aspartyl-L-proline								-0.209467
Caproic acid		0.224522		-0.230295		-0.228942		0.23344
Choline				-0.220947				
cis-Aconitic acid	Martin et al 2015 (Supp Table 2)				Martin et al 2015 (Supp Table 2)	0.289111		-0.215474
Creatinine	Martin et al 2015 (Supp Table 2)					-0.242136		
Cysteine			-0.233847				0.208435	
Decanoylcarnitine							0.296667	-0.256962
Deoxycorticosterone				-0.228152	Martin et al 2015 (Supp Table 2)			
Epinephrine glucuronide							0.203595	
Fumarate								-0.209495
Galactaric acid						0.203425		
Galacturonic acid						0.268785		
Gamma-Glutamylcysteine		0.235754						
Glyceryl-glycoside		0.236335						
Glycine						0.353263		
Guanosine			-0.25528					
Hexanedioic acid					Martin et al 2015 (Supp Table 2)	0.226237	0.229783	-0.288115

Hexanoylglycine							0.344164	
Hydroxyphenylacetylglycine		-0.25681		0.237248				
Indolelactic acid			-0.216899					
Isocitrate	Martin et al 2015 (Supp Table 2)							-0.207257
Isohomovanillic acid		0.226966		-0.2173	Martin et al 2015 (Supp Table 2)			
L-anthionine			-0.297686	0.21044				
L-Aspartyl-L-phenylalanine		-0.240737		0.263526				
Maleic acid		0.223461	0.269736	-0.319652				
Mannitol	Martin et al 2015 (Supp Table 2)				Martin et al 2015 (Supp Table 2)	0.329475		
N-Glycyl-L-Proline						0.330147		-0.258
N-Methyl-D-aspartic acid							0.202348	
Norepinephrine sulfate				0.210555				
Pantothenic acid							0.229916	-0.213286
Paraxanthine			-0.273963	0.261325				
p-Cresol			0.220543					
Phenylacetylglycine							0.258677	
p-Hydroxymandelic acid	Martin et al 2015 (Supp Table 2)		0.229459	-0.26346				
Proline						0.270308		-0.238382
Pseudouridine	Martin et al 2015 (Supp Table 2)				Martin et al 2015 (Supp Table 2)		0.228864	
Quinolinic acid							0.27287	-0.251699
Rhamnose						0.220647	0.252399	-0.299229
Ribitol	Martin et al 2015 (Supp Table 2)				Martin et al 2015 (Supp Table 2)	0.311007		
Ribonic acid	Martin et al 2015 (Supp Table 2)						0.235562	

Ribose	Martin et al 2015 (Supp Table 2)	0.246473			Martin et al 2015 (Supp Table 2)			
Serine					Martin et al 2015 (Supp Table 2)	0.313499		
Sorbose					Martin et al 2015 (Supp Table 2)	0.228465		
Succinate					Martin et al 2015 (Supp Table 2)	0.218721		
Succinylacetone		-0.257808						
Threitol					Martin et al 2015 (Supp Table 2)		0.249672	
Tryptophan						0.242844		-0.240216
Tyrosine			0.219523				0.201738	
Uric acid	Martin et al 2015 (Supp Table 2)				Martin et al 2015 (Supp Table 2)		-0.210708	0.231175
Urocanic acid	Martin et al 2015 (Supp Table 2)		-0.243818					
Plasma Metabolites	**U-tAs Associated**	**%iAs Associated**	**%MAs Associated**	**%DMAs Associated**	**U-tAs Associated**	**%iAs Associated**	**%MAs Associated**	**%DMAs Associated**
(R*,R*)-2,3-Dihydroxybutanoic acid		-0.217614	-0.285698	0.32592		-0.247464		
11,14-Eicosadienoic acid			-0.231819					
11-Eicosenoic acid						0.233489		
1H-Indole-3-acetamide		-0.276774	-0.235141	0.331153				
1-Hydroxycyclohexen			-0.303929	0.302109				
1-Monooleoylglycerol							0.232453	-0.217388
2,3,4-Trihydroxybutyric acid							-0.247694	0.209861
2,5-dihydroxybenzoic acid		-0.24575						
2-Ethyl-3-hydroxypropionic acid						-0.284817		

2-furanylmethanol				0.22427			
2-Hydroxy-3-methylbutyric acid			-0.265957	0.287551	0.290309		
2-hydroxybutanoic acid			-0.242539	0.268472			
2-Hydroxyglutaric acid					0.243172		
2-Oxo-4-methylvaleric acid			-0.267228	0.281755			
2-Oxoglutaric acid						-0.210545	
3,4-Dehydro-DL-proline					-0.176301		
3-Aminosalicylic acid		-0.271217		0.279703			
3-hydroxyanthranilic acid		-0.215782			0.239452		-0.233026
3-Hydroxybutyric acid						0.259151	-0.233626
3-Hydroxyhippuric acid		-0.221225			0.290367		-0.276859
3-Hydroxyvaleric acid						0.214464	
3-Methyl, 3-hydroxybutanoic acid		-0.343266		0.350963			
3-methyl-2-oxovalerate				0.203824			
3-Methylthiopropionic acid	Martin et al 2015 (Supp Table 2)		-0.243882	0.270416			
4-Deoxypyridoxine		-0.257441			0.222566		-0.219599
4-Methyl-2-oxovaleric acid			-0.249396	0.276688			
6-Deoxy-mannose		-0.259659		0.249709			
8-Hydroxy-7-methylguanine				0.213461			
Alanine			-0.251836	0.291148			
Alloisoleucine		-0.269428	-0.284764				
Aminomalonic acid					0.203242		-0.233933

Arabitol		-0.268956		0.27191			
Arachidonic acid			-0.208688	0.235272			
Aspartic acid						-0.340247	0.235421
Creatinine						0.23473	
Cystine							0.197624
Dihomo-gGamma-Linolenic acid			-0.288582	0.29584			
Dihydrothymine					-0.224611		
Dihydrouracil		-0.228029			0.272431		-0.208977
Ethanolamine						-0.242329	
Fructose						-0.354731	
Gluconic acid, lactone		-0.317014		0.294934			
Glucopyranose		-0.222477		0.256088			
Glucose			-0.317237	0.303442			
Hexanoic acid		-0.339242		0.323567			
Hydroxyacetic acid		-0.220953	-0.246988	0.3032		-0.229232	
Hypotaurine						-0.283522	
Hypoxanthine						-0.27749	0.247696
Indoleacrylic acid			-0.334643	0.297617			
Isoleucine			-0.231031				
Kynurenine				0.240393			
Lanthionine					-0.268581		0.276356
Leucine			-0.278066	0.312821			
LysoPC(16:0)			-0.216071	0.358799			
Mannose		-0.210802					
Matitol						-0.271016	0.212655
Methyl Phosphate				0.242029			
Monoethyl phthalate	Martin et al 2015 (Supp Table 2)				0.226049		-0.210587
Myo-Inositol, phosphate			-0.285732	0.248448			
N-Acetylglutamine						-0.205141	
N-formyl-glycine		-0.221746		0.236345			
N-methylleucine		-0.221787		0.256592		0.213534	

Nonanoic acid	-0.244775					
Octanoic acid				0.243331		
Orotidine				-0.257117		0.222009
Phenylalanine		-0.32971	0.296083			
Phosphoenol pyruvic acid		-0.226821				
Phosphohydroxypyruvic acid				0.218277		
p-Hydroxyphenylacetic acid					-0.226094	0.223962
Propane-1,2-diol	-0.370685		0.345704			
Pseudo uridine	-0.216404					
Ribofuranose	-0.329472		0.262901			
Succinate		-0.236397				
Taurine		-0.235806				
Threose						-0.199407
trans-9-Octadecenoic acid						-0.197996
Tryptophan		-0.341143	0.303779			
Tyrosine		-0.194192	0.234835			
Uridine			0.227272			
Valine	-0.22065	-0.292817	0.332665			

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